XENOBIOTIC-DEPENDENT REGULATION OF GENE EXPRESSION IN HUMAN PLACENTAL CELL LINES

BY

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This dissertation is dedicated to Yukun, and also to my parents, brothers and sisters.

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KEY TO ABBREVIATIONS

AD	actinomycin D
Ah	aryl hydrocarbon
AhR	aryl hydrocarbon receptor
Arnt	aryl hydrocarbon receptor nuclear translocator
α-NF	α-naphthoflavone
BaP	benzo(*a*)pyrene
BPDE	benzo(*a*)pyrene-7,8-diol-9,10-epoxide
BSA	bovine serum albumin
c-myc	celluar myc gene or ribonucleic acid
c-Myc	c-myc oncoprotein
cDNA	complementary deoxyribonucleic acid
CHX	cycloheximide
CYP1A1	cytochrome P 450 1A1
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRE/XRE	dioxin responsive element/xenobiotic responsive element
DTT	dithiothreitol
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
HCB	2,2',4,4',5,5'-hexachlorobiphenyl
hCG	human chorionic gonadotropin

hr	hours
IGF-II	insulin-like growth factor II
IGFBP	insulin-like growth factor binding protein
kb	kilobases
k_d	dissociation constant
kDa	kilodaltons
MMP	matrix metalloproteinase
MNF	3'-methoxy-4'-nitroflavone
mRNA	messenger ribonucleic acid
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PCB	polychlorinated biphenyl
PCDF	polychlorinated dibenzofurans
PDGF	platelet-derived growth factor
PMSF	phenyl-methyl sulfonyl fluoride
RNA	ribonucleic acid
SE	standard error
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCB	3,3',4,4'-tetrachlorobiphenyl
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
t-PA	tissue plasminogen activator
u-PA	urokinase plasminogen activator

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This study evaluated the usefulness of human placental trophoblastic choriocarcinoma cell lines BeWo and JEG-3 to study the effects of the prototype environmental chemicals on the expression of important trophoblast growth regulatory genes. The environmental chemicals studied are the aryl hydrocarbon (Ah) receptor agonists, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo(*a*)pyrene (BaP). The placental genes under study include the growth factors, transforming growth factor (TGF)-α, TGF-β1 and the epidermal growth factor (EGF) receptor, the proteinase inhibitor, plasminogen activator inhibitor (PAI)-2, and the protooncogene, c-myc. This study first demonstrated that both cell lines possess a functional Ah receptor system as evidenced by CYP1A1 induction following TCDD and BaP exposure. Secondly, induction of CYP1A1 does not appear to be directly linked with loss of EGF receptor. BaP decreased EGF receptor binding and protein in both cell lines, without affecting EGF receptor mRNA level. TCDD, however, was not found to alter the EGF receptor expression in either cell line. Thirdly, significant dysregulation of TGF-β1 and c-myc gene expression was

observed with BaP but not TCDD in JEG-3 cells. BeWo cells primarily showed effects of both chemicals on hCG secretion, BaP on TGF-β1 and TCDD on TGF-α expression. Finally, BaP has been shown to inhibit choriocarcinoma cell proliferation and invasion, whereas TCDD increased cell invasion without affecting cell proliferation.

In summary, BaP-mediated changes in EGF receptor, TGF-β1 and c-myc expression were correlated with altered trophoblast proliferation and invasiveness. These altered processes may underlie mechanisms by which xenobiotics such as those found in cigarette smoke disrupt placental function and lead to fetal growth retardation. Data also indicate that TCDD and BaP produce placental toxicity through different mechanisms. In addition, this study supports the feasibility of using the BeWo and JEG-3 cell lines to investigate biomarkers and mechanisms of placental toxicity.

CHAPTER I
INTRODUCTION

Human Placenta: A Primary Target Organ of CYP1A1 Inducers

Maternal cigarette smoking during pregnancy has been associated with increased incidence of spontaneous abortions, congenital malformations and fetal growth retardation (Pirani, 1978; Naeye, 1980; Sachs, 1989; Alderman et al., 1994; Gabriel, et al., 1994; Handler et al., 1994). Changes in placental epidermal growth factor (EGF) receptors (Wang et al., 1988; Gabriel, et al., 1994), amino acid uptake (Rowell, 1981) and endocrine function (Mochizuki et al., 1984) have been reported in association with maternal cigarette smoking. Cigarette smoke contains a number of biologically active compounds (Hoffmann et al., 1978), but it is not clear which of those compounds are responsible for fetoplacental toxicity. It has been reported that maternal cigarette smoking induced placental cytochrome P450 1A1 (CYP1A1) activity (Sesardic et al., 1990), produced smoking-related covalent DNA adducts in human placenta (Everson et al., 1986), and increased sister chromatid exchange frequency in cytotrophoblasts (Shulman et al., 1991). In this regard, a major polycyclic carcinogen present in the cigarette smoke is benzo(*a*)pyrene (BaP), an inducer of CYP1A1 and a DNA-damaging agent (Hoffmann et al., 1978; Kaiserman and Rickert, 1992). Study of primary human placental cells found that exposure to BaP directly resulted in an alteration in amino acid uptake (Guyda, 1991) and a loss of EGF receptor binding and autophosphorylation (Guyda et al., 1990). The selective alterations in EGF, but not insulin, receptors in the placentas of women who smoked have been linked with fetal growth retardation in these pregnancies (Gabriel et al., 1994). Thus, smoking-related

fetotoxicity may involve direct effects of cigarette smoke on the placenta which may be mediated by BaP and related compounds.

As observed with infants of cigarette smoking mothers, low birth weights were also observed in infants born to mothers who consumed polychlorinated biphenyl (PCB)/dibenzofuran (PCDF)-contaminated rice oil or fish during pregnancy (Sunahara et al., 1987; Lindstrom et al., 1995). Further study has found that decreased birth weights in infants following *in utero* exposure to PCBs/PCDFs were associated with decreased functional EGF receptors in the placentas (Sunahara et al., 1987). CYP1A1 activity was also found to be markedly induced in PCB/PCDF-exposed placentas (Wong et al., 1986). These studies clearly indicate that human placental CYP1A1 activity is highly inducible by environmental xenobiotic exposure and the human placenta is a primary target organ of PCBs/PCDFs, a large group of halogenated hydrocarbons represented by its prototype 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Furthermore, animal studies have shown that gestational exposure to TCDD or PCB mixtures can result in fetotoxicity and teratogenicity without overt toxic effects on the mother (McNulty, 1985; Golub et al., 1991; Rier et al., 1993), suggesting that the potential mechanisms of fetotoxicity of the CYP1A1 inducers may involve direct effects of these chemicals on the placenta.

The Objective of This Study

This research was undertaken to further investigate molecular mechanisms by which the prototype CYP1A1 inducers, TCDD and BaP, have disruptive effects on placental function. It is well known that the induction of CYP1A1 is via the cytosolic aryl hydrocarbon (Ah) receptor, which is a ligand-dependent transcription factor. Substantial evidence indicates that TCDD and related Ah receptor ligands act as endocrine disruptors and growth modulators by persistently altering the expression of a battery of genes. However, little is known regarding the Ah receptor regulated genes in human placental cells. Our hypothesis is that TCDD and BaP alter placental function by disrupting local

autocrine and paracrine networks which are recognized to be important for the regulation of trophoblast proliferation, differentiation and invasiveness. The objective of this project was to identify the cellular and molecular processes altered by TCDD and BaP in human placental cells in an effort to develop an *in vitro* model for the study of placental toxicity of environmental chemicals.

Cultured Placental Cells: An *In Vitro* Model for the Study of Fetoplacental Toxicity

Study of the human fetoplacental toxicity of environmental chemicals has been difficult because of a lack of sufficient quantities of early pregnancy tissue, as well as the fact that placental trophoblasts in primary culture do not actively proliferate (Lewis et al., 1994). It is essential, therefore, to explore the use of alternate models of proliferative human trophoblasts.

The human trophoblastic cell lines BeWo and JEG-3, derived from gestational choriocarcinoma have been shown to retain many characteristics of normal trophoblasts and display a number of the functional features of differentiated syncytiotrophoblasts (Pattillo et al., 1968; Kohler and Bridson, 1971; Hochberg et al., 1991). Both cell lines have been used as a model for in vitro studies of regulation of trophoblastic gene expression and placental endocrine function (Kato and Braunstein, 1991; Chiang and Main, 1994; Matsuo and Strauss III, 1994), although their validity as a placental model has not been established. Our interest is in the feasibility of using cultured placental cell lines as a placental system to investigate the potential toxicity of environmental chemicals. We have chosen BeWo and JEG-3 cell lines as a model system to investigate the events which occur during Ah receptor-mediated CYP1A1 induction in human placental trophoblasts.

The next four sections of this Chapter present a brief review of the current understanding of the mechanisms of action of TCDD and BaP, Ah-responsive genes and trophoblast growth control networks.

General Characterization of the Mechanisms of Action of TCDD and BaP

Ah Receptor

It is generally believed that the biochemical and toxic effects of TCDD and related halogenated aromatics are mediated by the Ah receptor, a cytosolic protein and a ligand-activated transcription factor (Whitlock Jr., 1993; Hankinson 1995; Fernandez-Salguero et al., 1996). The Ah receptor is distinct from the steroid receptor family in two notable ways. First, there is no known endogenous physiological ligand for the Ah receptor; second, the Ah receptor is a basic-helix-loop-helix protein, whereas the steroid receptors are zinc-finger proteins (Whitlock Jr., 1993; Dolwick et al., 1993; Hankinson 1995). The known ligands for the Ah receptor are foreign planar aromatic compounds, including polycyclic aromatic hydrocarbons represented by BaP, as well as halogenated aromatic compounds whose prototype is TCDD. A strong correlation exists between the binding affinity of various ligands to the Ah receptor, the potency of these chemicals as a CYP1A1 inducer, and their ability to produce various toxic responses (Safe, 1990). The current view of the mechanism by which the Ah receptor regulates gene transcription is that the binding of specific ligands to this receptor induces a biochemical and conformational change which converts the protein into an active DNA binding form (Whitlock Jr., 1993; Hankinson 1995).

Northern blot analysis indicates that the human Ah receptor mRNA is expressed in all tissues examined, which include liver, lung, heart, kidney, brain, skeletal muscle and placenta (Dolwick et al., 1993). It is worth noting that placental tissue is one of the richest known sources of Ah receptor in humans (Manchester et al., 1987; Dolwick et al., 1993). Importantly, the high levels of Ah receptor in human placenta may account for the great sensitivity of this tissue to receptor agonists, such as those found in cigarette smoke and PCB-contaminated rice oil (Wong et al., 1986; Lucier et al 1988). A recent study demonstrates that the extracts of urban air and vehicle exhaust particulates contain

significant amounts of Ah receptor binding activity, the majority of which is attributed to unidentified polycyclic aromatic hydrocarbons (Mason, 1994). In light of the high level of Ah receptor expression, placenta is regarded as a very sensitive tissue for investigation of the potential human risk of exposure to environmental polycyclic aromatic pollutants.

Metabolic Activation

Although both BaP and TCDD bind to the Ah receptor and induce CYP1A1 activity, only BaP is metabolized by CYP1A1 into a series of reactive metabolites. The latter can cause teratogenicity (Legraverend et al., 1984), immunotoxicity (Kong et al., 1994) and hematotoxicity (Holladay and Smith, 1994; Zhu et al., 1995), as well as DNA damage resulting in mutagenicity and carcinogenicity (Levin et al, 1978). In contrast, TCDD is resistant to metabolism and all, or at least most, of the TCDD-induced biologic effects are mediated by TCDD-Ah receptor complex via regulation of a battery of structural genes (Greenlee et al., 1990; Huff et al., 1994). In this regard, BaP may be more toxic than TCDD because the toxic effects of BaP can be amplified and exacerbated by its reactive metabolites. It is postulated that the carcinogenicity of TCDD may result from alterations in the DNA-damaging potential of some endogenous compounds, as well as disregulation of cellular differentiation and/or division by an initial interaction of TCDD with the Ah receptor (Huff et al., 1994). Therefore, our comparative study of BaP- and TCDD-mediated effects on related endpoints in the same cell line will further our knowledge of the importance of initial interaction of ligands with the Ah receptor and metabolic activation in the mechanisms of action of BaP- and TCDD-type carcinogens.

EGF Receptor: A Possible Marker of Placental Toxicity

The EGF receptor is a well-characterized 170 kDa single polypeptide transmembrane glycoprotein which is detectable in a wide variety of tissues *in vivo* and cell lines in culture (Adamson, 1990). Human placenta shows a high level of expression of

EGF receptor which is localized in the proliferative cytotrophoblasts in very early placenta, and subsequently in mitotically inactive differentiated syncytiotrophoblasts as gestation advances (Ladines-Llave et al., 1991). There is an increase in the number and binding capacity of EGF receptors in the trophoblast with advancing normal gestation (Carson et al., 1983; Lai and Guyda, 1984; Adamson, 1990). EGF has been shown to stimulate trophoblast proliferation and endocrine function (Lai and Guyda, 1984; Morrish et al., 1987; Maruo et al., 1992). Thus, evidence strongly supports a physiological role for the EGF receptor system in normal fetoplacental growth and development throughout pregnancy.

Study of placentas from cigarette smokers and nonsmokers found that EGF-stimulated receptor kinase activity was markedly decreased in placental membrane proteins from smokers (Lucier et al., 1987; Wang et al., 1988). In this regard, BaP is a potent polycyclic carcinogen that is present in the particulate phase of cigarette smoke (Hoffmann et al., 1978; Kaiserman and Rickert, 1992). Our study of human placental cells in primary culture found that exposure to BaP directly resulted in a dose-dependent selective loss of EGF receptor binding activity and autophosphorylation, which was greatest in cells from first trimester placentas (Guyda et al., 1990). It is significant that the original observation of selective alterations in EGF, but not insulin, receptors in the placentas of women who smoked was recently confirmed by Gabriel *et al.* (1994), and further shown to be linked with intrauterine growth retardation in these pregnancies. A separate study of women who were exposed to polychlorinated biphenyl-contaminated rice oil found that birth weights were decreased in infants following *in utero* exposure in association with decreased placental EGF receptor tyrosine kinase activity (Lucier et al., 1987; Sunahara et al., 1987). These and other reports (Fujita et al., 1991; Fondacci et al., 1994) provide substantial evidence that EGF receptors are altered in placental membranes from women whose fetuses show intrauterine growth retardation. Thus alterations in EGF receptor might provide a good biomarker of effect for fetoplacental toxic xenobiotics. In comparison with previous

studies in normal trophoblasts, the present study of EGF receptors in choriocarcinoma cells provides information on similarities and differences between normal and transformed trophoblast cells in response to xenobiotics, as well determines the validity of the choriocarcinoma cells as a model system for placental toxicity studies.

TCDD-responsive Growth Control Genes

At present, the best characterized response to TCDD is the transcriptional regulation of the CYP1A1 gene. It is well known that transcriptional activation involves the binding of the liganded Ah receptor to several DNA recognition sites known as xenobiotic-responsive elements (XREs) or dioxin-responsive elements (DREs) in upstream enhancer regulatory regions of the CYP1A1 gene (Greenlee et al., 1990; Whitlock Jr., 1993; Hankinson, 1995). The receptor-XRE interaction increases the accessibility of the downstream promoter and hence activates CYP1A1 transcription. Thus, the binding of the liganded Ah receptor to the DNA recognition sites in upstream enhancers is a pivotal event in the mechanism of TCDD action. Theoretically, any gene that contains 5'-regulatory regions highly homologous to the XREs of the CYP1A1 gene may be regulated by the TCDD-Ah receptor complex. Dependent upon tissue or gene specific factors, the expression of XRE-containing genes can potentially be induced or suppressed by the liganded Ah receptor complex. Increasing evidence indicates that TCDD alters the expression of a number of genes important in cell growth and differentiation, including the EGF receptor, transforming growth factor (TGF)-α, EGF, TGF-βs, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), plasminogen activator inhibitor 2 (PAI-2), urokinase-type plasminogen activator (u-PA), tissue plasminogen activator (t-PA), cathepsin D (an aspartyl protease), and the estrogen receptor (Hudson et al., 1985; Gierthy et al., 1987; Abbott and Birnbaum, 1989, 1990a and 1990b; Astroff et al., 1990; Choi et al., 1991; Lin et al., 1991; Safe et al., 1991; Sutter et al., 1991; Gaido et al., 1992; Gaido and Maness, 1994; Sewall and Lucier, 1995; Vogel and Abel, 1995).

One of the hallmarks of the effects of TCDD on growth control genes is significant tissue- and species-specificity. TCDD decreases EGF receptor binding activity in human keratinocytes and mouse liver without affecting the amount of mRNA for the EGF receptor (Hudson et al., 1985; Lin et al., 1991). In contrast, EGF receptor mRNA was reported to be decreased by TCDD exposure in rat uterus and liver in correlation with the loss of EGF binding (Astroff et al., 1990; Sewall et al., 1995). TCDD has also been shown to reduce the number of EGF receptors in hepatic plasma membrane of rat, guinea pig, mouse and hamster, with different degrees of sensitivity and potency (Madhukar et al., 1984). It warrants note, however, that TCDD has also been reported to stimulate EGF receptor expression and proliferation in the mouse embryonic palate and ureter epithelial cells (Abbott and Birnbaum, 1989 and 1990a).

Studies with both normal and transformed human keratinocytes have shown that TCDD increased the steady-state mRNA levels for TGF-α, IL-1β and PAI-2 (Choi et al., 1991; Sutter et al., 1991; Gaido et al., 1992; Gaido and Maness, 1994), and suppressed the mRNA expression for TGF-β2 (Gaido et al., 1992; Gaido and Maness, 1994), whereas TGF-α and PAI-2 mRNAs were not altered by TCDD in rat liver (Vanden Heuvel et al., 1994). Moreover, one study demonstrated that the induction of TGF-α expression in transformed SCC-12F keratinocytes by TCDD occurs post-transcriptionally by increased mRNA stabilization, while TGF-β2 expression is reduced due to a decrease in the rate of TGF-β2 gene transcription (Gaido et al., 1992). There are alterations in protein levels of TGF-α, TGF-β2, IL-1β and PAI-2 which correlate with altered mRNA levels following TCDD exposure in nontransformed human keratinocytes (Gaido and Maness, 1994). u-PA protein levels were induced in these cells, however, the mRNA level for u-PA was not altered following TCDD treatment (Gaido and Maness, 1994).

A recent study with the human breast cancer cell line MCF-7 has shown that the mRNA levels of TGF-α, TGF-β3, IL-1β and TNF-α were increased by TCDD, whereas the TGF-β1 and TGF-β2 mRNAs were unchanged (Vogel and Abel, 1995). This study

further reported that the enhanced secretion of TGF-β was accompanied by an inhibition of cell growth by TCDD (Vogel and Abel, 1995). Furthermore, TCDD has been reported to reduce the expression of TGF-α, EGF and TGF-β1 in mouse palatal epithelial and mesenchymal cells (Abbott and Birnbaum, 1990b). However, it is important to emphasize the following: 1) TGF-α mRNA is not affected in mouse (Lin et al., 1991) and rat (Vaden Heuvel et al., 1994) liver, and increased in human keratinocytes (Choi et al., 1991; Gaido et al., 1992; Gaido and Maness, 1994) and breast cancer cells (Vogel and Abel, 1995) by TCDD; 2) TGF-β1 mRNA is not changed by TCDD in either human keratinocytes (Gaido et al., 1992) or breast cancer cells (Vogel and Abel, 1995).

Studies in MCF-7 mammary tumor cells indicate that TCDD reduces estrogen-induced secretion of t-PA and cathepsin D (Gierthy et al., 1987; Safe et al., 1991). A further study showed that the expression of hepatic and uterine estrogen receptor protein was decreased by TCDD in mice (Devito et al., 1992). It has also been observed that TCDD caused a decrease in nuclear estrogen receptor levels in MCF-7 and wild-type Ah-responsive Hepa 1c1c7 cells, but was inactive in Ah non-responsive mutant Hepa 1c1c7 cells (Safe et al., 1991). In this regard, the human estrogen receptor gene has been shown to contain a DNA sequence that binds activated mouse and human Ah receptors, providing a possible mechanism for transcriptional regulation of the estrogen receptor by TCDD (White and Gasiewiz, 1993).

Altogether, patterns of growth control genes may be differentially altered by TCDD in separate species, tissues and/or cells. Modulation of gene expression by TCDD results in differential changes in either mRNA or protein level for specific genes and can occur by multiple mechanisms. These mechanisms may include transcriptional control, posttranscriptional modulation of mRNA, translational control and posttranslational modulation of protein. Our effort to identify the important TCDD-responsive growth regulatory genes in placental cells serves to add more knowledge to how growth control

genes respond to TCDD differently in various tissues, and also increases our understanding of tissue-specific responsiveness of TCDD.

Trophoblast Growth Control Factors

Human trophoblast proliferation, differentiation and invasiveness is regulated by local autocrine/paracrine networks. The major networks involve 1) the growth factors, TGF-βs, TGF-α/EGF and their common receptor, the EGF receptor (Frolik et al., 1983; Filla et al., 1993; Amemiya et al., 1994), 2) the cytokine, IL-1β (Kauma et al., 1990), 3) the protooncogenes, e. g. c-myc and c-sis (Goustin et al., 1985), and 4) the proteases and their inhibitors such as u-PA and its inhibitor PAI-2 (Feinberg et al., 1989; Hofmann et al., 1994).

TGF-α

TGF-α is widely expressed in a variety of normal adult cells, embryos and fetuses (Lee et al., 1995). The mature 50 amino acid form of human TGF-α is synthesized as an internal part of a transmembrane 160 amino acid precursor (Derynck et al., 1984; Wong et al., 1989; Anklesaria et al., 1990; Lee et al., 1995). Not only the mature secreted form but also the membrane-anchored precursor binds to the EGF receptor, leading to signal transduction and cellular response (Derynck et al., 1984; Lee et al., 1995). Notably, TGF-α is found in maternal decidual cells, villous and extravillous cytotrophoblasts, and villous syncytiotrophoblasts throughout pregnancy (Filla et al., 1993; Lysiak et al., 1993; Horowitz et al., 1993), which is colocalized with its receptor, the EGF receptor (Ladines-Llave et al., 1991; Filla et al., 1993). The proliferation of first trimester human trophoblast cells in culture is stimulated by exogenous TGF-α (Lysiak et al., 1993 and 1994) . In a study with choriocarcinoma cell lines JEG-3 and Jar, Lewintre et al. (1994) found that TGF-α elevates the levels of mRNA, protein and catalytic activity of 17β-hydroxysteroid

dehydrogenase type 1 that catalyzes the reversible interconversion of estrone and estradiol. This study suggests that TGF-α may play a role in estrogen production in the human placenta.

In light of the above evidence, TGF-α appears to have an autocrine, paracrine/juxtacrine role in trophoblast growth and function. Together with evidence that TGF-α is differentially regulated by TCDD in human keratinocytes (Choi et al., 1991; Gaido et al., 1992, Gaido and Maness, 1994) and breast cancer cells (Vogel and Abel, 1995), mouse embryonic palatal cells (Abbott and Birnbaum, 1990b) and liver (Lin et al, 1991), and rat liver (Vanden Heuvel et al., 1994), it is of common interest to both the developmental biologist and the toxicologist as to whether the expression of the TGF-α gene in human placental cells is altered by xenobiotics.

TGF-β1

One of the initial sources for purification of TGF-β to homogeneity is the human placenta (Frolik et al., 1983). All tissues found at the maternal-fetal interface, including first-trimester decidua, placenta, and placental membrane, contain TGF-β and express TGF-β1 mRNA (Kauma et al., 1990). In this regard, TGF-β immunoreactive protein is localized in the cytoplasm of villous syncytiotrophoblast and extravillous trophoblast cells throughout gestation and TGF-β1 mRNA is expressed in both syncytiotrophoblasts and cytotrophoblasts (Lysiak et al., 1995), suggesting that trophoblast cells themselves can regulate their own invasiveness in an autocrine manner. TGF-β derived from decidua and trophoblasts is the prime mediator in the control of invasion by first trimester trophoblasts (Lala and Graham, 1990; Graham and Lala, 1991). A mechanism by which TGF-β1 acts to control invasion is through reduction in collagenase type IV activity which parallels increased expression of tissue inhibitor of metalloproteinases in trophoblasts (Lala and Graham, 1990; Graham et al., 1994). A second mechanism by which TGF-β1 blocks

trophoblast invasion is by inhibition of proliferation and enhanced differentiation of human trophoblast cells into noninvasive syncytiotrophoblasts (Graham et al., 1992).

In view of the major regulatory roles of TGF-β1 in trophoblast proliferation, differentiation and invasiveness, it is meaningful to explore whether xenobiotics disrupt TGF-β1 expression and subsequently alter placental autocrine/paracrine growth control networks.

c-Myc

c-Myc is a well known nuclear oncoprotein, a sequence-specific transcription factor, which regulates a variety of genes important in normal cellular proliferation and differentiation processes (Vastrik et al., 1994). During human placental development, the pattern of c-Myc expression is closely linked to the highly proliferative and invasive phenotype of cytotrophoblasts (Goustin et al., 1985; Maruo and Mochizuki, 1987). The nonproliferative differentiated syncytiotrophoblasts have not been found to display detectable levels of c-Myc expression (Ohlsson, 1989). Cultured first trimester trophoblasts respond to PDGF with elevated levels of c-myc mRNA and protein expression, accompanied by an activation of DNA synthesis (Goustin et al., 1985). Thus, studies indicate that c-Myc plays a role in normal trophoblast proliferation and placental development.

Not surprisingly, c-myc mRNA is constitutively highly expressed in choriocarcinoma cells (Nachtigal et al., 1992; Arbiser et al., 1993). Its expression can be suppressed by the chemotherapeutic drug methotrexate (MTX) in both BeWo and JEG-3 cells (Nachtigal et al., 1992; Arbiser et al., 1993). In this regard, MTX has been shown to be able to induce these choriocarcinoma cells to change their usual cytotrophoblastic phenotype and acquire morphologic and functional characteristics typical of intermediate trophoblasts (Taylor et al., 1991; Nachtigal et al., 1992). Altogether, these results reveal an inverse relationship between c-myc mRNA expression and trophoblast differentiation.

Further studies of the effects of TCDD and BaP on c-myc gene expression and choriocarcinoma cell proliferation may increase our understanding of interactions between protooncogene expression and trophoblast growth.

PAI-2

Mammalian embryonic development and growth require implantation of the blastocyst into the uterus. During hemochorial placentation, characteristic of humans and rodents, embryonic trophoblast cells invade through the uterine epithelium and deep into the maternal stroma. Invasion of trophoblasts requires cell surface-associated proteolysis that is potent but properly controlled (Lala and Graham, 1990). Of interest, PAI-2, the primary inhibitor of u-PA, has been suggested to play a role in the controlled invasion of the maternal decidua by trophoblasts during human implantation (Feinberg et al., 1989; Hofmann et al., 1994). In particular, PAI-2 has been immunohistochemically colocalized with u-PA and PAI-1 in cytotrophoblasts, intermediate trophoblasts and syncytiotrophoblasts at the maternal-fetal interface in early human implantation sites (Hofmann et al., 1994), with PAI-2 being the predominant PAI in villous syncytiotrophoblasts in term human implantation sites (Feinberg et al., 1989). Plasma PAI-2 levels are undetectable in nonpregnant women, but increase progressively in normal pregnancy, and then decrease dramatically soon after delivery (Kruithof et al., 1987), likely reflecting the completion of the placental function. Decreased plasma levels of PAI-2 have been found to be associated with intrauterine growth retardation, suggesting an impaired placental and fetal growth (Boer et al., 1988; Estelles et al., 1991; Lindoff and Astedt, 1994). Thus, evidence has shown that PAI-2 is an important regulatory protein of normal implantation and a possible marker of placental growth.

Interestingly, characterization of the promoter 5'-regulatory region of PAI-2 gene (Kruithof and Cousin, 1988) has revealed a XRE core sequence (5'-TNGCGTG-3') that provides the recognition motif for the Ah receptor (Sutter et al., 1991). In this regard,

TCDD exposure has been shown to stimulate the expression of PAI-2 mRNA in human keratinocytes, primary hepatocytes, monocytic cells, and hepatoma and breast cancer cells (Sutter et al., 1991; Gaido and Maness, 1994; Gohl et al, 1996; Dohr et al, 1995). Therefore, it is of great interest to determine whether the expression of PAI-2, a possible placental function marker, is regulated by TCDD or BaP in human placental cells.

CHAPTER 2
MATERIALS AND METHODS

Materials

Chemicals and Bioreagents

BaP, cycloheximide and actinomycin D were obtained from the Sigma Chemical Co. (St. Louis, MO), and TCDD from Midwest Research Institute (Kansas City, MO) through the National Cancer Institute Chemical Carcinogen Reference Repository. α-naphthoflavone (α-NF) was from Eastman Kodak Co. (Rochester, NY), 3,3',4,4'-tetrachlorobiphenyl (TCB) from RFR, Corp. (Hope, RI), and 2,2',4,4',5,5'-hexachlorobiphenyl (HCB) from Ultra Scientific (North Kingstom, RI). 3'-methoxy-4'-nitroflavone (MNF) was kindly provided by Dr. Stephen Safe (Texas A&M University, College Station, TX). Recombinant human EGF was purchased from Genzyme (Cambridge, MA). ^{125}I-protein A, [α-^{32}P]dCTP and [α-^{32}P]UTP were obtained from ICN Pharmaceuticals Inc (Costa Mesa, CA), and ^{125}I-EGF and [^{3}H-methyl] thymidine from Amersham Life Sciences (Arlington Heights, IL). The prime-It® II random primer labeling kit and NucTrap® probe purification columns were obtained from Stratagene (La Jolla, CA). CellTiter 96™ non-radioactive cell proliferation assay kit was obtained from Promega (Madison, WI), and the Fisher Diagnostics LeukoStat stain kit was purchased from Fisher Scientific (Pittsburgh, PA). The FastTrack mRNA isolation kit was from Invitrogen Corporation (San Diego, CA). Oligo(dT) cellulose, cell culture media and antibiotics were from Gibco/BRL (Grand Island, NY), and fetal bovine serum from Hyclone Laboratories (Logan, UT). All other chemicals were reagent or molecular biology grade and were obtained from standard commercial sources.

Recombinant cDNA Clones

Plasmids containing DNA for EGF receptor (pE7), TGF-α (phTGF1-10-3350), TGF-β1 (phTGFB-2), c-myc (pG1-5'-c-myc), CYP1A1 (phP1-450-3') and β-actin (HHCI89; 65129) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The plasmids containing cDNA for Ah receptor (phuAhR, Dolwick et al., 1993), Arnt (pBM5/Neo-M1-1, Hoffman et al., 1991) and PAI-2 (clone 18, Sutter et al., 1991) were kindly provided by Dr. Christopher Bradfield (Northwestern University, Chicago, IL), Dr. Oliver Hankinson (University of California at Los Angeles, Los Angeles, CA) and Dr. William Greenlee (University of Massachusetts, Worcester, MA), respectively. The probes used were a 2.7 kb *SmaI* fragment for Ah receptor, a 2.6 kb *BamHI* fragment for Arnt, a 1.0 kb *EcoRI* fragment for CYP1A1, a 2.4 kb *ClaI* fragment for EGF receptor, a 3.3 kb *EcoRI* fragment for TGF-α, a 2.1 kb *EcoRI* fragment for TGF-β1, a 2.1 kb *BstZI* fragment for PAI-2, a 1.6 kb *SacI* fragment for c-myc and a 1.1 kb *EcoRI* fragment for β-actin. All the probes are human cDNAs, except the c-myc probe which is human genomic DNA.

Antibodies and ELISA Kits

The polyclonal sheep anti-human EGF receptor and rabbit anti-human c-myc antisera were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), and the polyclonal goat anti-rat CYP1A1 antiserum from Gentest (Woburn, MA). The rabbit biotinylated anti-sheep IgG was from Vector Laboratories Inc. (Burlingame, CA), while horseradish peroxidase labeled goat anti-rabbit IgG and rabbit anti-goat IgG were from Bio-Rad Laboratories (Hercules, CA). The Micro-Elisa total βeta-hCG test kit was purchased from Leinco Technologies Inc. (St. Louis, MO), and the TGF-β1 ELISA system from Promega (Madison, WI).

Methods

Cell Cultures and Chemical Treatment

Human placental choriocarcinoma cell lines BeWo and JEG-3 were obtained from ATCC. BeWo cells were cultured in Ham's F-12 medium supplemented with 15% FBS, and JEG-3 cells in Eagle's minimum essential medium supplemented with 10% FBS, respectively, in a humidified atmosphere containing 5% CO_2 at 37°C. All complete media were supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml). Cells were grown to confluency and media changed every 2 to 3 days. Confluent cells were subcultured after trypsinization. Unless otherwise indicated, all experiments were initiated when cells were at approximately 40-60% confluence. Cells were cultured in the absence or presence of various chemicals, added either in DMSO or ethanol with final concentration of the vehicle being 0.1%, for the various times as indicated. The appropriate vehicle was added to control cultures. For RNA and protein analysis, cells were cultured in duplicate or triplicate for each experiment point. For proliferation assays, cells were cultured in triplicate or quadruplicate.

EGF Binding Assay

Cells were washed three times with PBS to remove BaP or TCDD and then incubated with 100 pM ^{125}I-EGF in the presence or absence of unlabeled EGF for 90 min at room temperature or 5 hr at 4°C. After careful rinsing to remove unbound radioactivity, the cells were solubilized in 0.5 N NaOH and the total binding of ^{125}I-EGF was determined by gamma counting. Specific binding was expressed as the difference between radioactivity bound in the absence (total binding) and presence (nonspecific binding) of excess unlabeled EGF (100 nM). For Scatchard analysis, cells were incubated with increasing concentrations of ^{125}I-EGF (1.25 to 200 pM) for 1.5 hr at room temperature. Nonspecific binding of ligand was measured by adding excess unlabeled EGF to cultures

for each concentration of ^{125}I-EGF. Each point on the Scatchard plot represents specific binding of ^{125}I-EGF.

Western Immunoblot Analysis

General procedure. Cells were rinsed two to three times, collected by scraping with a rubber policeman, and lysed in 1 ml PBS using three freeze-thaw cycles. The total cell membrane fraction was obtained by centrifugation at 12,000*g* for 10 min at 4°C and resuspended in PBS. Alternatively, cells were scraped into lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4) and phenyl-methyl sulfonyl fluoride (PMSF), and 1μg/ml aprotinin, leupeptin and pepstatin. The lysate was transferred to a microcentrifuge tube using a syringe fitted with a 21 gauge needle, incubated on ice for 30-60 min, and centrifuged at 12,000*g* for 15 min at 4°C. The supernatant liquid is the total cell lysate. Samples of membrane or total cell lysate protein (100 μg) were then separated by 7.5% or 10% SDS-PAGE and transferred electrophoretically to nitrocellulose filters using 25 mM Tris, 192 mM glycine buffer at pH 8.2, with or without 20% methanol according to the procedure of Towbin *et al.* (1979).

CYP1A1 protein. The blotted nitrocellulose sheet was blocked for 20 min in 3% gelatin in 20 mM Tris, containing 500 mM NaCl, pH 7.5, and then incubated with goat anti-rat CYP1A1 (1:500 dilution) or preimmune goat serum for 2 hr, followed by peroxidase conjugated anti-goat IgG for 1 hr as described previously (Wang et al., 1988). The immunoreactive protein was visualized by incubation with 3-amino-9-ethylcarbazole in the presence of 0.015% hydrogen peroxide, and quantitated by optical density scanning using a Microtek ScanMaker II scanner and NIH image program.

EGF receptor protein. The blotted nitrocellulose filters were washed in 100 mM Tris containing 0.1% (v/v) Tween 20 and 0.9% NaCl, pH 7.5 (TTBS) for 30 min, and

then incubated sequentially with polyclonal anti-human EGF receptor antiserum (diluted to 1 μg/ml in TTBS) or preimmune sheep serum for 60 min, followed by biotinylated anti-sheep IgG for 60 min, and Vectastain ABC reagent for 30 min. Immunoreactive bands were visualized and quantitated as described above. Alternatively, quantitation of immunoreactive EGF receptor protein was carried out by a modification of the method of Gargosky *et al.* (1992). In brief, nitrocellulose filters were blocked with 1% (w/v) BSA in TTBS for 18 hr at 4°C, then incubated sequentially with sheep anti-EGF receptor antiserum or preimmune sheep serum, rabbit anti-sheep IgG, and ^{125}I-protein A. The immunoreactive bands were quantitated by scanning cpm using a βetascope 603 blot analyzer, after which filters were exposed to X-Omat film at -80°C for 12 to 18 hr for autoradiography.

c-Myc protein. The blotted nitrocellulose sheet was blocked for 30 min in 5% fat-free dried milk in PBS, and then incubated with rabbit anti-human c-myc protein (0.5 μg/ml) or preimmune rabbit serum overnight at 4°C, followed by peroxidase conjugated goat anti-rabbit IgG for 1.5 hr at room temperature. The blots were washed thoroughly with deionized water after removing the primary and secondary antibodies, respectively. The immunoreactive protein was visualized by enhanced chemiluminescence detection system (Amersham Life Sciences, Arlington Heights, IL) and Kodak X-Omat film, according to the manufacturer's instructions, and the band quantitated by densitometry as described above.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from cultured cells by acid guanidinium thiocyanate phenol-chloroform extraction according to Xie and Rothblum (1991). Poly $(A)^+$ RNA was prepared by the method of Celano et al (1993). Alternatively, poly $(A)^+$ RNA was extracted directly from the cells using FastTrack mRNA isolation kits, according to the manufacturer's instructions (Invitrogen). For Northern blotting, 40 μg of total cellular

RNA or 10 μg of poly $(A)^+$ RNA was denatured, fractionated in 1% agarose formaldehyde gel and transferred to nitrocellulose or nylon membranes. The probes were labeled with [α-^{32}P]dCTP using a random primer DNA labeling kit. Prehybridization was carried out in 50% formamide containing 5 X Denhardt's solution, 4 X SSC (20 X SCC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1% (w/v) SDS, 40 mM sodium phosphate, and 0.25 mg/ml yeast RNA at 42°C overnight. The hybridization was performed at 42°C for 20 to 40 hr in the same buffer but containing 1 X Denhardt's solution and the ^{32}P-probes as indicated. The filter was washed twice in 2 X SSC/0.1% SDS at room temperature for 30 min, then twice in 0.2 X SSC/0.1% SDS at 42°C for 30 min, and once at 0.1 X SSC/0.1% SDS at 65°C for 15 min. Alternatively, hybridization of RNA with the indicated DNA probes was carried out 2 to 4 hr at 68°C in ExpressHyb hybridization solution as instructed (CLONTECH Laboratories, Inc., Palo Alto, CA). The presence of specific RNA was detected by autoradiography, after which, filters were stripped and reprobed with each of the indicated DNA probes. Hybridization signals were quantitated by densitometry with the each message standardized to the β-actin transcript.

Nuclear Run-off Assay

The nuclear run-off transcription assay was performed as described by Greenberg and Ziff (1984) with slight modifications. Briefly, 100 μl nuclei (2 X 10^7) collected by Nonidet P-40 lysis were added to 100 μl 2 X reaction buffer (10 mM Tri-HCl, pH 8, 5 mM $MgCl_2$, 300 mM KCl, 5 mM dithiothreitol, 1 mM ATP, CTP and GTP), and 100 μCi of [α-^{32}P]UTP followed by incubation at 30°C for 45 min. After degradation of the DNA by 10 U/ml of RNase-free DNase I (Boehringer Mannheim), the nascent transcripts were isolated by acid guanidinium thiocyanate phenol-chloroform extraction, and hybridized with nylon blots with 2 μg of specific c-myc, TGF-β1, CYP1A1 and β-actin cDNA immobilized in each dot. The hybridization was processed in 2 ml of 500 mM sodium phosphate buffer, pH 7.2, 7% SDS for 48 h at 65°C. After an initial wash in 250 mM

sodium phosphate buffer containing 1% SDS, the blots were washed twice in 100 mM sodium phosphate buffer containing 1% SDS, 15 min each at 65°C. The specific hybridization signal was detected by autoradiography and quantitated by densitometry.

mRNA Stabilization Assay

Cells were treated with 10 μM BaP or 0.1% DMSO for 24 hr, and actinomycin D at 5 μg/ml was added to the media. At the indicated times after addition of actinomycin D, total RNA was isolated and processed for Northern analysis, and the hybridized signal was quantitated by densitometry as described above.

ELISA for Secreted Proteins

Conditioned media were clarified by passing through a 0.45-μm filter, and protein inhibitors were added to a final concentration of 2 μg/ml pepstatin A, 1 μg/ml leupeptin, 2 μg/ml aprotinin and 1 mM PMSF. The conditioned media were stored at -70°C or -20°C until use.

TGF-β1 assay. The specific detection of biologically active TGF-β1 in the media was carried out with a TGF-β1 ELISA system (Promega), according to the manufacturer's direction. Briefly, the latent form TGF-β1 in the media was activated by adding 1 μl of 1 N HCl to 50 μl sample medium. Maxi-Sorp (high protein affinity) 96 well ELISA plates were coated with monoclonal anti-TGF-β1 overnight at 4°C, blocked and incubated with the acid-activated sample media or TGF-β1 standard for 1.5 hr at room temperature. After washing for 5 times with wash buffer, the plates were incubated with polyclonal anti-TGF-β1 for 2 hr at room temperature, and washed as described above, followed by incubation with a species-specific horse radish peroxidase conjugated antibody for 2 hr at room temperature. The unbound conjugate was removed by washing, and the specifically bound TGF-β1 was detected using a chromogenic substrate. The extinction at 450nm was then

recorded using an ELISA plate reader, which is directly proportional to the amount of biologically active TGF-β1 in the test sample.

hCG assay. Assay of hCG in the media was carried out with a Micro-Elisa total βeta-hCG test kit (Leinco Technologies, Inc.). Briefly, the test sample was allowed to react simultaneously with the coated and conjugated antibodies, resulting in the hCG molecule being sandwiched between the solid phase and enzyme-linked antibodies. After a 30 min incubation at room temperature, the sample well was washed thoroughly to remove free enzyme-labeled antibody. An enzyme substrate-chromogen was added to the well and incubated for 15 min at room temperature resulting in the development of a blue color. The addition of 1.0 N H_2SO_4 converted the color to yellow. The extinction at 450nm was then recorded, which is directly proportional to the concentration of hCG in the sample.

Cell Proliferation Assay

MTT assay. Cell proliferation was determined using the tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) MTT dye assay according to the CellTiter 96™ non-radioactive cell proliferation assay procedure (Promega). This assay is based on the cellular conversion of the tetrazolium salt into a formazan product that is quantitated using an ELISA plate reader. Direct comparisons between ^{3}H-thymidine incorporation and the MMT assay have shown less than a 5% difference between assays for determination of growth-factor concentrations. Briefly, cells were subcultured for 20 hr in 96-well tissue culture plate at a density of 5.0 X 10^3/well for BeWo and 2.5 X 10^3/well for JEG-3, and exposed to various compounds for 48 hr in the presence or absence of FBS, followed by a 4 hr incubation with MTT dye. After solubilization of the formazan product, the extinction at 595nm was recorded with 690nm as reference wavelength using an ELISA plate reader. Relative cell proliferation was determined by comparing the extinction with that of control cells.

[^{3}H]Thymidine incorporation assay. Cells were cultured at 5.0 X 10^4 cells/well in 24-well plates for 20 hr and treated with various concentration of TCDD or BaP for 5 days, or 10 nM TCDD and 10 μM BaP for various times as indicated. Medium was changed every 24 to 48 hr. Cells were transferred to serum-free medium 20 hr before the addition of tritiated thymidine and pulsed with 1 μCi/ml [^{3}H]thymidine for the last 3 hr. After trypsinization, cells were harvested onto glass fiber filter strips with a cell harvester, and incorporated radioactivity was determined by liquid scintillation counting. Replicate cultures were harvested for cell number counting. The cell number was counted using a hemocytometer.

In Vitro Invasion Assay

The Matrigel invasion assay was performed using a two compartment Boyden Chamber (Terranova et al., 1986). Briefly, subconfluent cell cultures were incubated with BaP or TCDD for 48 hr. Cells were collected and resuspended in complete media to a density of 2.0 X 10^5 cells/ml after trypsinization. Twenty-eight microliters of the cell suspension, in the presence of the respective chemicals, were added to the lower compartment of the Boyden chamber. The lower compartment was overlaid with a matrigel-coated porous (8 μm diameter pores) polyvinyl-pyrrolidone-free polycarbonate membrane (Nuclepore, Pleasanton, CA), and the upper compartment fastened on. The chamber was inverted and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 2 to 3 hr, allowing the cells to attach to the membrane. The chamber was next placed upright and 50 μl media without chemicals was added to the upper compartment. After incubation for 18 hr, the membrane was removed and scraped free of uninvaded cells on the lower side of the membrane. The cells that invaded through the membrane were stained with LeukoStat stain set and counted under a microscope.

Protein Assay

Protein content of membrane suspensions and total cell lysates was determined by the method of Bradford (1976) using BSA as a standard.

Data Analysis

All experiments were performed using duplicate to quadruplicate cultures at each concentration of EGF, BaP, TCDD and/or time point. A one-factor ANOVA was employed to assess dose effects followed with Fisher's protected least significant difference (PLSD) test when a significant dose effect ($p < 0.05$) was detected in the ANOVA. A two-factor ANOVA was employed to analyze the BaP dose effect, the EGF treatment effect and the potential interactive effect on cell proliferation. An unpaired Student's *t*-test was also used to analyze the data. All statistical analyses were performed with the Macintosh StatView512+™ or MicrosoftExcel program.

CHAPTER 3
EVIDENCE THAT THE HUMAN PLACENTAL CELL LINES BEWO AND JEG-3 HAVE A FUNCTIONAL AH RECEPTOR SYSTEM

Introduction

Evidence indicates that TCDD-induced toxicities are initiated by activation of the Ah receptor system. The Ah receptor is a ligand-activated transcription factor that functions in partnership with the Ah receptor nuclear translocator (Arnt) protein. Upon binding of a ligand, the Ah receptor undergoes a series of biochemical changes which include dissociation of hsp90 proteins, translocation to the nucleus, and dimerization with the Arnt protein. The heterodimer of the liganded-Ah receptor and Arnt proteins binds to upstream enhancer region specific DNA sequences termed XREs/DREs, resulting in the activation of a battery of genes, including drug metabolizing enzymes and growth regulatory factors, which ultimately lead to the observed biologic response (Whitlock Jr., 1993; Hankinson, 1995; Safe, 1995). The response requires both the Ah receptor and the Arnt proteins. TCDD fails to activate CYP1A1 transcription in Ah receptor-defective cells and in Arnt-defective cells (Whitlock Jr., 1993; Hankinson, 1995). Therefore, potential success in the exploration of TCDD-regulated genes is highly dependent on the choice of an appropriate cell model which not only expresses both Ah receptor and Arnt gene products, but also possesses a known inducible positive control gene. To date, the best characterized response to TCDD and related Ah receptor ligands is the transcriptional activation of the CYP1A1 gene. Figure 3-1 summarizes the current understanding of the molecular mechanism of induction of the CYP1A1 gene expression.

An earlier study with JEG-3 cells observed that aryl hydrocarbon hydroxylase, an enzyme activity associated with CYP1A1, was induced by TCDD and a benzothiazole

derivative 2-(4'-chlorophenyl)benzothiazone; however, no detectable immunoreactive CYP1A1 protein was observed by Western analysis in the JEG-3 cells following exposure to these chemicals (Kärenlampi et al., 1989). Thus this study was not conclusive as to whether the CYP1A1 gene in JEG-3 cells is inducible by Ah receptor agonists. Since this report, no study has established that the induction of aryl hydrocarbon hydroxylase is truly through the induction of CYP1A1 gene expression in JEG-3 cells, nor is any information available regarding the Ah receptor system in BeWo cells. It is, therefore, necessary to determine whether the BeWo and JEG-3 cells have a functional Ah receptor system. The results presented in this Chapter provide direct evidence that the BeWo and JEG-3 cells express Ah receptor and Arnt mRNA and are able to form a functional Ah receptor complex following Ah receptor agonist stimulation.

Results

Expression of Ah Receptor and Amt mRNA in BeWo and JEG-3 Cells

We initially examined the Ah receptor, Arnt and CYP1A1 steady state mRNA levels in control, TCDD and BaP-treated cells. Northern blot analysis revealed the presence in both BeWo and JEG-3 cells of a single Ah receptor transcript of 6.6 kb and three Amt transcripts of 4.2, 2.6 and 1.8 kb (Figure 3-2), as has been reported in human placental tissues as well as in human liver, HepG2 and mouse Hepa-1 cell lines (Hoffman et al., 1991; Dolwick et al., 1993). As shown in Figure 3-2, both Ah receptor and Arnt mRNA transcripts were constitutively expressed. BeWo cells had a lower steady state level of Ah receptor mRNA than JEG-3 cells, while the Arnt mRNA was abundant and comparable in both cell lines. TCDD or BaP treatment at 48 hr had little effect on the steady state mRNA levels for Ah receptor and Arnt.

Unlike the Ah receptor and Arnt mRNA, CYP1A1 mRNA transcript was undetectable in either BeWo or JEG-3 control cells by Northern blot analysis. A 3.0 kb

CYP1A1 mRNA, however, was highly induced by TCDD and BaP (Figure 3-2), indicating the ability of the BeWo and JEG-3 cells to form a functional Ah receptor complex following exposure to the Ah receptor ligands, as previously reported in human keratinocytes and mouse hepatoma cells (Gaido et al., 1992; Israel et al., 1985).

Superinduction of CYP1A1 mRNA and Structure-Activity Specificity

Treatment of JEG-3 cells with 10 µg/ml cycloheximide (CHX), a protein synthesis inhibitor, for 24 h either in the presence or absence of Ah receptor ligands resulted in substantial increases in CYP1A1 mRNA (Figure 3-3). The induction of CYP1A1 mRNA in cells treated with TCDD or BaP plus CHX was much greater than that in cells treated with TCDD, BaP or CHX alone. Thus, a superinduction was observed in choriocarcinoma cells treated simultaneously with the Ah receptor ligands and the protein synthesis inhibitor, which is a well-known property of Ah receptor-mediated induction of CYP1A1 mRNA in hepatoma cells (Whitlock Jr., 1993; Hankinson, 1995; Safe, 1995).

Moreover, treatment with the PCB congener 3,3',4,4'-tetrachlorobiphenyl (TCB), a weak Ah receptor ligand, resulted in a weak induction of CYP1A1 mRNA, while 2,2',4,4',5,5'-hexachlorobiphenyl (HCB), a non-Ah receptor ligand, was unable to induce any CYP1A1 mRNA expression in JEG-3 cells (Figure 3-3). This congener specificity is consistent with previous observations on structure-activity relationships for binding to the Ah receptor (Safe, 1990 and 1992). The observed superinduction and sterospecificity support the involvement of the Ah receptor.

Induction of CYP1A1 Protein

Western immunoblot analysis showed that a 55 kDa immunoreactive CYP1A1 protein was induced by TCDD and BaP in a concentration dependent manner in both BeWo and JEG-3 cells (Figure 3-4). In addition, figure 3-4 indicates that induction of CYP1A1 is a sensitive marker for exposure to as low as 0.1 nM TCDD or 1 µM BaP, and was

maximal at 10 nM TCDD or 10 μM BaP in both cell lines. Insofar as 100 μg of cell protein was applied to each lane, the intensities of the respective lanes indicate that more immunoreactive CYP1A1 protein was present in JEG-3 cells, compared with BeWo cells, which is correlated with the higher level of CYP1A1 mRNA induction in the JEG-3 cells (Figure 3-2).

Discussion

The present study for the first time demonstrated that the human placental trophoblastic choriocarcinoma cell lines BeWo and JEG-3 expressed both Ah receptor and Arnt mRNA, and CYP1A1 mRNA was highly inducible by the two prototype Ah receptor ligands, TCDD and BaP (Figure 3-1 and 2). The induced CYP1A1 mRNA is expressed as CYP1A1 protein was induced by TCDD and BaP (Figure 3-4). These data contrast with the previous report that no immunoreactive CYP1A1 protein was detected by Western analysis in JEG-3 cells following TCDD treatment (Kärenlampi et al., 1989). One possible reason is that the antibody (Mab 1-7-1, mouse anti-rat liver P450) previously used may not recognize the specific epitopes of the CYP1A1 proteins in JEG-3 cells. In addition, the present results indicate that CYP1A1 mRNA and protein are induced by TCDD and BaP to a greater extent in JEG-3 cells than in BeWo cells and are correlated with the higher level expression of the Ah receptor mRNA in JEG-3 cell line.

The present finding that CYP1A1 mRNA was superinduced by simultaneous treatment of TCDD or BaP with CHX is in agreement with the previous studies in human keratinocytes (Gaido et al., 1992), human breast cancer cells MCF-7 and MDA-MB-231 (Arellano et al., 1993), Hepa 1c1c7 mouse hepatoma cells (Israel et al., 1985), and quail aortic smooth muscle cells (Ou and Ramos, 1995). The mechanism of the superinduction is not fully understood. It has been shown that the CHX-mediated superinduction of CYP1A1 required a functional Ah receptor complex (Israel et al., 1985) and involved multiple DNA-binding factors that interact with the XREs/DREs (Saatcioglu et al., 1990).

It is now generally agreed that CHX inhibits the synthesis of a labile dominant repressor which competes with the liganded Ah receptor complex and superinduction ensues (Whitlock Jr., 1993; Hankinson, 1995).

The finding that the CYP1A1 mRNA in JEG-3 cells was slightly induced by the PCB congener TCB is consistent with previous reports that TCB is 100-times less potent than TCDD in binding to the Ah receptor and inducing CYP1A1 expression. In contrast, the observation that HCB congener does not induce CYP1A1 mRNA is consistent with previous reports that HCB does not bind to the Ah receptor or induce CYP1A1 expression (Safe, 1990 and 1992). The sterospecificity further supports the role of the Ah receptor in the induction of CYP1A1 mRNA.

In conclusion, the placental cell lines BeWo and JEG-3 possess a functional Ah receptor system, and respond to TCDD and BaP directly with an induction of CYP1A1 mRNA and protein expression. The induction of the CYP1A1 gene expression exhibits structure-activity specificities in response to PCB congeners with different binding affinities to the Ah receptor, and superinduction occurs following simultaneous treatment with TCDD or BaP plus CHX. These characteristics are consistent with well-documented responses of other cells to the Ah receptor ligands (Israel et al., 1985; Safe, 1990; Sutter et al., 1991; Gaido et al., 1992; Arellano et al., 1993; Whitlock Jr., 1993; Hankinson, 1995; Ou and Ramos, 1995). Thus, data indicate that the BeWo and JEG-3 cells are suitable for the investigation of the events which occur during Ah receptor-mediated CYP1A1 induction in human placental trophoblastic cells.

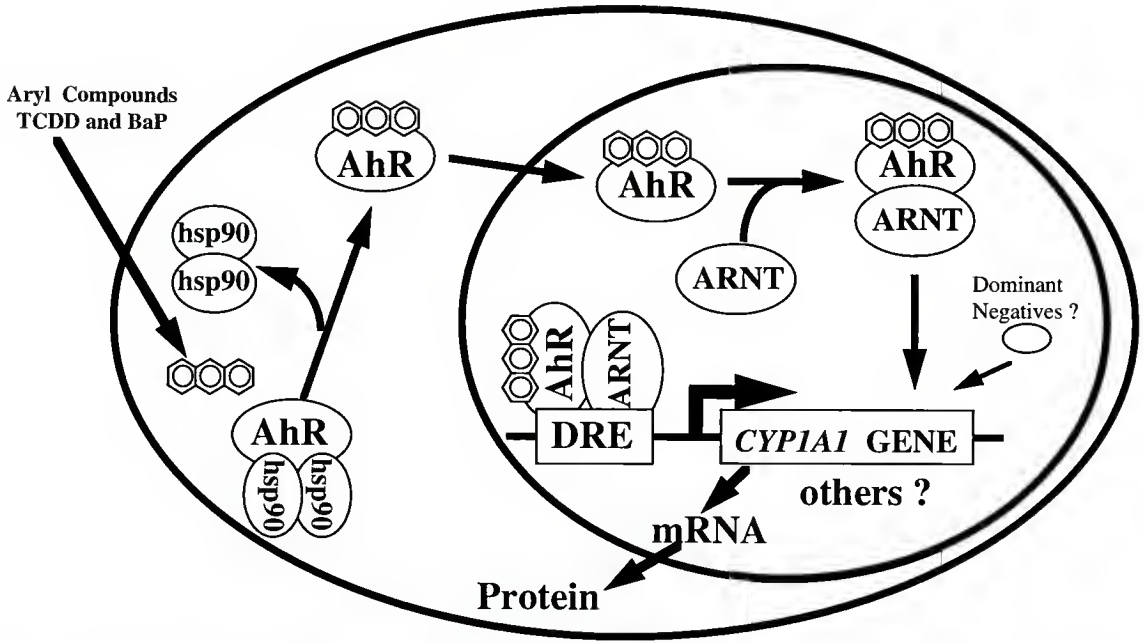


Figure 3-1. Molecular mechanism of ligand-induced CYP1A1 gene transcription. Modified from Whitlock Jr. (1993) and Hankinson (1995). The unliganded Ah receptor is a soluble intracellular protein, and associates with other proteins such as hsp90. The binding of polycyclic or halogenated aromatic ligands leads to dissociation of the Ah receptor from hsp90, followed by reassociation with Arnt. The heterodimer Ah receptor-Arnt complex acquires a high affinity for specific DNA sequences termed XRE/DRE. The receptor heterodimer-XRE/DRE interaction in responsive genes increases accessibility of the gene promoter and initiates transcription.

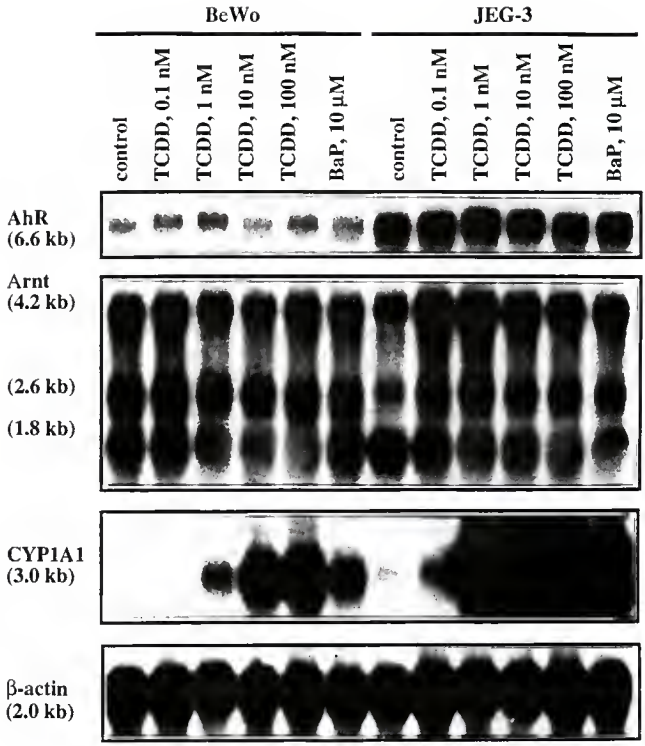


Figure 3-2. Northern blot analysis of Ah receptor, Arnt and CYP1A1 mRNA. Cells were incubated with or without TCDD and BaP for 48 hr. Poly(A)$^+$RNA, 10 µg, was denatured, blotted, and hybridized with ^{32}P-labeled cDNA probes as described in Materials and Methods.

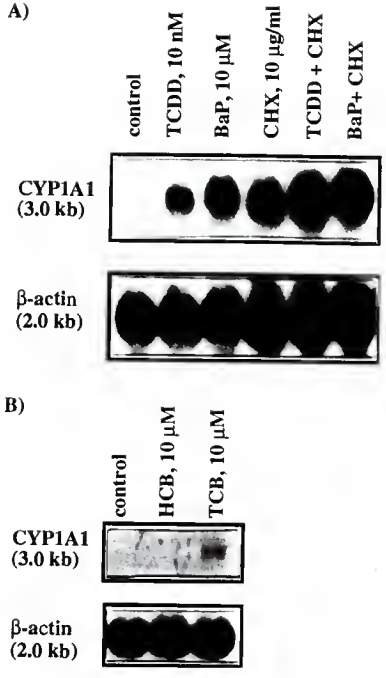


Figure 3-3. Northern analysis of CYP1A1 mRNA. A) Superinduction of CYP1A1 mRNA in the presence of CHX; B) Induction of CYP1A1 mRNA by two PCB congeners, TCB, a weak Ah receptor agonist, and HCB, a non-Ah receptor agonist. Cells were treated with 10 nM TCDD, 10 μM BaP, 10 μg/ml CHX, TCDD plus CHX, and BaP plus CHX for 24 hr, or 10 μM TCB and HCB for 48 hr. Total RNA, 40 μg, was denatured, fractionated, transferred, and hybridized sequentially with CYP1A1 and β-actin probes.

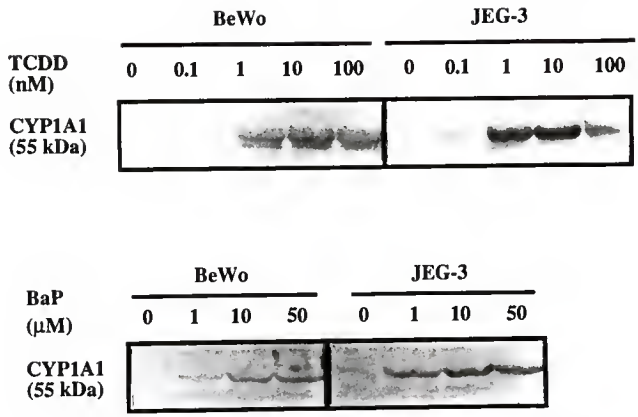


Figure 3-4. Effects of TCDD and BaP on immunoreactive CYP1A1 proteins. The BeWo and JEG-3 cells were harvested following incubation with TCDD or BaP for 48 hr. Total cell protein, 100 μg, was separated by 10% PAGE, transferred to nitrocellulose, and immunostained with anti-CYP1A1 antibody.

CHAPTER 4
EFFECTS OF TCDD AND BAP ON EGF RECEPTOR EXPRESSION

Introduction

Human placenta shows a high level of expression of EGF receptor which is colocalized with its ligands EGF and TGF-α in trophoblasts throughout gestation (Ladines-Llave et al., 1991; Filla et al., 1993), suggesting a physiological role for the EGF receptor system in normal fetoplacental growth and development. Substantial evidence indicates that EGF receptors are altered in placental membranes from women whose fetuses show intrauterine growth retardation (Lucier et al., 1987; Sunahara et al., 1987; Wang et al., 1988; Fujita et al., 1991; Fondacci et al., 1994; Gabriel et al., 1994). In this regard, maternal cigarette smoking or exposure to PCB/PCDF mixtures has been associated with decreased EGF receptor tyrosine kinase activity in the placenta, which has been further shown to be linked with intrauterine growth retardation in these pregnancies (Lucier et al., 1987; Gabriel et al., 1994). It has been proposed that alterations in EGF receptor may be a good placental biomarker of exposure to toxic halogenated aromatic compounds (Lucier et al., 1987).

Several known inducers of CYP1A1, including TCDD and BaP, have been shown to downmodulate EGF receptor expression in various species, tissues and cell lines (Ivanovic and Weinstein, 1982; Kärenlampi et al., 1983; Madhukar et al., 1984; Hudson et al., 1985; Astroff et al., 1990; Guyda et al., 1990; Lin et al., 1991; Sewall et al., 1993 and 1995). The mechanism of chemical-mediated EGF receptor downmodulation is not currently understood, neither is the mechanistic link between CYP1A1 induction, EGF receptor downmodulation, and chemical-mediated toxicity. The study presented in this

Chapter evaluated changes in EGF receptor number (B_{max}), binding affinity (K_d), internalization, and protein and mRNA content in the Ah-responsive choriocarcinoma cells BeWo and JEG-3 following TCDD and BaP treatment. A goal is to evaluate whether there is a dose-relationship between EGF receptor expression and CYP1A1 induction.

Results

Effects of TCDD and BaP on Specific Binding of ^{125}I-EGF to BeWo and JEG-3 cells

Scatchard analysis showed a single class of binding site with a K_d of 0.043 nM for BeWo and 0.048 nM for JEG-3 cells (Figure 4-1), which is similar to the high affinity binding site previously reported in human placental cells (Guyda et al., 1990) and human placental membranes (Lucier et al., 1987). Cultures treated with 10 nM TCDD for 48 hr did not show any changes in the B_{max} and K_d values in either cell line. In contrast, cultures treated with 10 μM BaP for 48 hr exhibited 34% and 42% decreases in the B_{max} in BeWo and JEG-3 cells, respectively, with little change in K_d values (Figure 4-1).

The next experiment evaluated whether the BaP-induced loss of EGF binding was mediated by internalization of cell surface receptors. Internalization of the EGF receptor in choriocarcinoma cells does not occur at 4°C as evidenced by the fact that more than 90% of the ^{125}I-EGF bound to both BeWo and JEG-3 cells was dissociated by an acid wash procedure which removes only surface bound ligand. ^{125}I-EGF-specific binding to BaP-treated cells was reduced to the same extent when binding was measured at 4°C or room temperature (Figure 4-2), while TCDD had no effect on the binding under either condition. These data indicate, therefore, that the BaP-related decrease in EGF binding is due to an alteration in available high affinity cell surface binding sites rather than internalization of the ligand.

The BaP-mediated decrease in EGF binding was time-dependent. The inhibition of EGF specific binding was observed within 6 hr following BaP treatment, which is similar to observations in human keratinocytes (Hudson, et al., 1985), mouse embryo fibroblasts (Ivanovic and Weinstein, 1982) and hepatoma cells (Kärenlampi et al., 1983). The inhibitory effect persisted at a constant level for at least 96 hr in BeWo cells, whereas the EGF specific binding progressively decreased in JEG-3 cells until 96 hr, the last time point examined (Figure 4-3). The sustained inhibitory effect of BaP on EGF binding is comparable to that previously reported with TCDD in human keratinocyte SCC-12F cells (Hudson et al., 1985).

Effects of TCDD and BaP on EGF Receptor Protein

The EGF receptor was detected by Western immunoblot as a 170 kDa band in the total cell membrane fraction of both BeWo and JEG-3 cells (Figure 4-4), which was comparable to the receptor species detected in human A-431 cells and rat liver microsomes (data not shown). The level of immunoreactive EGF receptor protein was significantly decreased following exposure to BaP in a concentration-dependent manner. In this regard, EGF receptor protein was decreased from control by 20, 41 and 52% in BeWo cells, and by 23, 58 and 53% in JEG-3 cells, following exposure to 1, 10 and 50 μM of BaP for 48 hr, respectively. These data provide further evidence that the reduced ^{125}I-EGF cell surface binding was due to the loss of total cellular EGF receptor protein. In contrast, exposure to TCDD for 48 hr did not alter the level of total cell EGF receptor immunoreactive protein in either cell line (Figure 4-4). The level of immunoreactive EGF receptor protein was 97, 106, 99 and 110% of control in BeWo cells, and 82, 85, 95 and 92% of control in JEG-3 cells, following exposure to 0.1, 1, 10 and 100 nM of TCDD for 48 hr, respectively.

In SCC-12F human keratinocyte cells the kinetics for the BaP- and TCDD-mediated decrease of EGF binding were found to be different (Hudson et al., 1985), such that

inhibition of EGF binding by BaP was maximal by 24 hr, whereas TCDD treatment for 72 hr was required to produce maximal inhibition. For this reason, we treated BeWo and JEG-3 cells with TCDD for longer time period and then determined the EGF receptor protein levels. No significant change in the EGF receptor protein level was observed following TCDD treatment for 24 to 96 hr in either cell line (Figure 4-5).

Relationship between CYP1A1 Induction and EGF Receptor Changes

A concentration-related decrease in the level of total cell EGF receptor immunoreactive protein was observed in association with the induction of CYP1A1 protein in both cell lines following BaP treatment (Figure 4-6). In contrast, exposure to TCDD for 48 hr caused no alteration in the level of EGF receptor protein in either cell line, despite the marked induction of the CYP1A1 protein. To determine whether the induction of CYP1A1 is required for BaP to decrease the EGF receptor protein content, we then studied the effects of the Ah receptor antagonists α-naphthoflavone (α-NF) and 3'-methoxy-4'-nitroflavone (MNF) on the BaP-mediated loss of EGF receptor protein. Although cotreatment of α-NF (1 and 10 μM) and TCDD partially antagonized the TCDD induced CYP1A1 protein expression in a concentration-related manner, cotreatment of JEG-3 cells with 10 μM of α-NF and 10 μM of BaP did not in any way inhibit the induction of CYP1A1 by BaP (Figure 4-7). An unexpected finding was that α-NF alone at 10 μM shared partial agonist activity with some induction of CYP1A1, as well as partial loss of EGF receptor protein. A similar observation has been reported with mouse Hepa-1 (Kärenlampi et al., 1983) and embryo fibroblast cells (Ivanovic and Weinstein, 1982) in which α-NF alone markedly decreased EGF binding. Thus, the partial agonist activity of α-NF in choriocarcinoma cells makes it difficult to make a conclusion about the role of CYP1A1 induction in BaP-mediated loss of EGF receptor.

A second Ah receptor antagonist, MNF, was also evaluated. MNF alone at 1 and 10 μM did not induce CYP1A1 mRNA in JEG-3 cells (Figure 4-8), and TCDD-induced

CYP1A1 mRNA expression was completely abolished by cotreatment with either 1 or 10 μM MNF. Thus MNF acted as a pure antagonist of TCDD action. However, MNF even at 10 μM was unable to antagonize the induction of CYP1A1 by 10 μM BaP, when added either 2 hr earlier or at the same time as BaP. In addition, BaP produced a comparable decrease in EGF receptor protein level in the absence or presence of MNF. Therefore, the results were again not definitive on whether there was a mechanistic link between CYP1A1 induction and EGF receptor loss in cells treated with BaP.

Effects of Actinomycin D and Cycloheximide on BaP-Mediated Changes in EGF Receptors

We next investigated whether BaP-mediated downmodulation of EGF receptor protein in JEG-3 cells requires the synthesis of other mRNAs and proteins, using the RNA synthesis inhibitor actinomycin D (AD) and the protein synthesis inhibitor cycloheximide (CHX). The presence of AD or CHX alone had no significant effect on EGF receptor protein levels after 16 to 24 hr exposure (Figure 4-9). When JEG-3 cells were treated simultaneously with 10 μM BaP and 5 μg/ml AD or 10 μg/ml CHX, both inhibitors completely blocked BaP-induced downmodulation of EGF receptor protein level. These data provide evidence that the BaP-mediated loss of EGF receptor was dependent on *de novo* mRNA and protein synthesis. In addition, induction of CYP1A1 protein was blocked by treatment with AD and CHX, and this was in association with the block of EGF receptor downmodulation in JEG-3 cells cotreated with BaP and AD or CHX. Thus data suggest that CYP1A1 activity or metabolism of BaP may be essential in the BaP-induced loss of EGF receptor protein.

Effects of TCDD and BaP on Steady State EGF Receptor mRNA Levels

The effect of TCDD and BaP on the steady state level of EGF receptor mRNA was analyzed by Northern blot techniques using a pE7 EGF receptor cDNA probe. BeWo and JEG-3 cells both showed two transcripts of 10 and 5.6 kb (Figure 4-10). Quantitation of

these bands further demonstrated that the steady state level of EGF receptor mRNA was not significantly changed by BaP or TCDD treatment in either cell line (Figure 4-11). These data suggest that the BaP-mediated decrease in EGF receptor protein does not involve changes in the steady state level of mRNA.

Discussion

Both BeWo and JEG-3 cells were found to express EGF receptor mRNA transcripts of 10 and 5.6 kb and express immunoreactive EGF receptor protein of 170 kDa, which is in agreement with values previously reported in human placenta and placental cell cultures (Wang et al., 1988; Guyda et al., 1990; Fujita et al., 1991; Gabriel et al., 1995). BaP treatment of both BeWo and JEG-3 cells resulted in a concentration-related decrease in the binding of ^{125}I-EGF, in agreement with earlier reports in primary cultures of early gestation human placental cells exposed to BaP (Guyda et al., 1990). Moreover, Scatchard analysis indicates a loss of high affinity EGF binding sites in choriocarcinoma cells following treatment with BaP. In this regard, dose-dependent loss of EGF binding has been previously reported with BaP in cultured human keratinocytes (Hudson et al., 1985), mouse embryo fibroblasts (Ivanovic and Weinstein, 1982) and hepatoma cell lines (Kärenlampi et al., 1983). In the choriocarcinoma cells, our data indicate that the reduced binding of ^{125}I-EGF to whole cells is not due to altered internalization of cell surface receptors because EGF binding was still significantly decreased at 4° when internalization is minimal. In addition, Western analysis confirmed that the decrease in binding of ^{125}I-EGF was associated with a loss of the EGF receptor protein following BaP treatment. These results further support our previous finding that the smoking-related defect in placental EGF receptor autophosphorylation appeared to be due to the loss of EGF receptor protein (Wang et al., 1988).

Although CYP1A1 inducers were found to decrease EGF binding in various cells more than a decade ago (Ivanovic and Weinstein, 1982; Kärenlampi et al., 1983; Madhukar

et al., 1984; Hudson et al., 1985), the mechanistic link between CYP1A1 induction and EGF receptor downmodulation still remains unclear. Structure-activity relationship and study of congenic Ah-responsive and -nonresponsive mouse strains have indicated a role of the Ah receptor in mediating EGF receptor downmodulation (Ivanovic and Weinstein, 1982; Lin et al., 1991; Safe, 1995). In mouse fibroblasts, Ivanovic and Weinstein (1982) found that exposure of BaP led to a time- and concentration-dependent decrease of EGF binding, whereas the highly reactive electrophilic metabolite of BaP, BaP-7,8-diol-9,10-epoxide (BPDE), did not significantly alter EGF binding. A comparison of a series of 16 polycyclic compounds further showed a correlation between the capacity to inhibit EGF binding and the apparent affinity of the same compound for the Ah receptor, as well as their ability to induce the CYP1A1 system (Ivanovic and Weinstein, 1982). In this regard, Lin *et al* (1991) reported that the Ah locus mediates the effects of TCDD on the hepatic EGF receptor in C57BL/6J mice.

The present study, however, found that TCDD resulted in a dose-dependent CYP1A1 induction without altering EGF receptor protein or binding, providing evidence that there is no causal relationship between CYP1A1 induction and EGF receptor downmodulation in choricarcinoma cells. These results clearly demonstrate that occupancy of the Ah receptor and/or interaction of the liganded-Ah receptor complex with the XRE DNA sequences *per se* does not affect EGF binding or EGF receptor protein level in choriocarcinoma cells, since both TCDD and BaP bind to the Ah receptor and induce CYP1A1 mRNA and protein, but only BaP alters EGF binding and EGF receptor protein level (Figure 4-1 to 9). On the other hand, these data imply that the loss of EGF binding and protein may be a consequence of metabolism of BaP to reactive metabolites, which does not occur with TCDD treatment due to its resistance to metabolism. In this regard, the most toxic BaP metabolite, BPDE, has been shown to effectively block EGF binding in the mouse hepatoma c4 mutant cell line which has a defective Ah receptor, as well as in the parent Hepa-1 cell cultures, whereas BaP itself decreased EGF binding only in Hepa-1

parent cells (Kärenlampi et al., 1983). In these experiments, TCDD was not found to affect the EGF binding in Hepa-1 cells. In addition, it is noteworthy that aryl hydrocarbon hydroxylase, an enzyme activity associated with CYP1A1, is highly inducible in JEG-3 cells by TCDD, providing evidence that JEG-3 cells have the capacity to metabolize BaP to reactive intermediates (Kärenlampi et al., 1989).

Studies with inhibitors of RNA and protein synthesis showed that block of CYP1A1 induction by AD or CHX also prevented the BaP-induced loss of EGF receptor protein, evidence which further supports that metabolism of BaP may be essential in the decrease of EGF receptor protein. However, these studies cannot exclude the possibility that another repressor protein may be involved in the downmodulation of the EGF receptor, particularly because neither AD nor CHX is a specific inhibitor of CYP1A1 mRNA or protein synthesis. In addition, data indicate that BaP-mediated loss of EGF receptors does not involve changes in the steady state mRNA level (Figure 4-11), suggesting that alterations in EGF receptor synthesis, protein processing and half life or modulation of autocrine networks by BaP are likely involved in EGF receptor downmodulation.

The Ah receptor activation pathway is a multistep process which involves the binding of agonist to the receptor, recruitment of the partner factor Arnt, and interaction of the liganded receptor-Arnt heterodimer with the target DNA XRE sequences. Disruption of any of these steps can block the Ah receptor-mediated induction of CYP1A1. The mechanism of antagonistic action of α-NF and MNF on Ah receptor function is not fully understood. Using Arnt-deficient cells and chimeric receptor techniques, Wilhelmsson et al. (1994) demonstrated that Ah receptor sequences rather than Arnt mediate the antagonistic effects of α-NF. At the same time, these authors found that the α-NF-occupied Ah receptor was able to recruit Arnt, resulting in XRE binding activity. In agreement with the experimental data, this study has shown that α-NF has dual functions with antagonism of TCDD induction of CYP1A1 when co-administered with TCDD, as well as action as a partial agonist in inducing CYP1A1 expression when administered

alone. The observed lack of antagonistic effect of α-NF on BaP induction of CYP1A1 is possibly due to their similar potency in stimulating Ah receptor function in JEG-3 cells.

MNF is a newly characterized pure Ah receptor antagonist in MCF-7 human breast cancer cells (Lu et al., 1995), in which cotreatment with 0.01 to 10 μM MNF plus 1 nM TCDD was reported to cause a concentration-dependent inhibition of TCDD-induced formation of the nuclear Ah receptor complex, CYP1A1 mRNA level and ethoxyresorufin O-deethylase activity. In the present study, coexposure of JEG-3 cells to MNF and TCDD completely blocked TCDD induction of CYP1A1 protein (Figure 4-8). No apparent antagonistic effect of MNF on BaP induction of CYP1A1 was observed, however, when JEG-3 cells were treated simultaneously with BaP and MNF. The absence of an antagonistic effect of MNF on BaP induction of CYP1A1 cannot be explained by their relative potencies in stimulating the Ah receptor function, particularly since MNF alone did not show any partial agonistic effect (Figure 4-8).

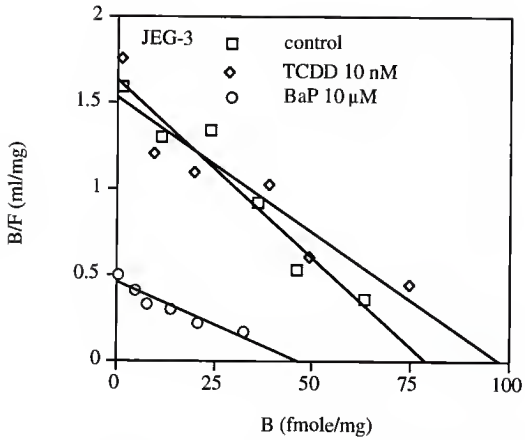
There is some evidence for multiple Ah receptor proteins. In addition to the well-characterized 8S Ah receptor, an intracellular 4S polycyclic hydrocarbon-binding protein has been reported to be involved in the induction of CYP1A1 in mouse hepatoma cells (Sterling et al., 1994). In this regard, evidence has shown that the Ah receptor complex exists as two distinct forms in MCF-7 human breast cancer cells (Lu et al., 1995) and in guinea pig hepatic cytosol following TCDD treatment (Swanson et al., 1993; Bank et al., 1995). Merchant et al. (1992) found that BaP bound to both the 4S binding protein and the Ah receptor, although the 4S binding protein was not required for the induction of CYP1A1. However, Manchester et al. (1987) found that only a specific Ah receptor binding peak rather than binding in 4 to 5S region was observed in sucrose density gradient profiles when human placental cytosols were incubated with [^{3}H]BaP. Moreover, our data in Chapter 3 (Figure 3-2) has shown that the higher level expression of the Ah receptor mRNA is directly correlated with a higher level induction of the CYP1A1 mRNA in JEG cells, compared with that in BeWo cells following BaP exposure (Figure 4-1).

Collectively, evidence strongly supports a mechanism in which BaP induces CYP1A1 via the Ah receptor. However, our finding that the pure Ah receptor antagonist MNF completely inhibited CYP1A1 induction by TCDD, but not by BaP, raises questions as to whether TCDD and BaP may induce CYP1A1 through different subtypes of the Ah receptor or through different forms of the DNA-binding Ah receptor complex.

The steady state level of mRNA for EGF receptors was not found to be significantly altered by either BaP or TCDD exposure in the BeWo and JEG-3 choriocarcinoma cell lines. In contrast, Fujita et al (1991) reported that the EGF receptor and its mRNA levels were decreased in placentas from pregnancies with intrauterine growth-retardation and diabetes mellitus. TCDD was associated with a reduction in both EGF binding and EGF receptor mRNA steady state levels in rat uterus (Astroff et al., 1990). However, exposure of human keratinocytes *in vitro* to TCDD and livers from TCDD-treated mice showed a reduction in maximal EGF binding without a change in the amount of mRNA for the EGF receptor (Lin et al., 1991). The observation that TCDD induces expression of TGF-α in keratinocytes (Choi et al., 1991) led to the hypothesis that increased expression of this peptide growth factor may lead to internalization of the EGF receptor and activation of cell responses. In recent studies, however, TGF-α mRNA was not found to be increased in rat liver by TCDD (Vanden Heuvel et al., 1994), whereas EGF receptor mRNA was reported to be decreased in correlation with the loss of EGF binding (Sewall et al., 1995). Finally, it warrants note that TCDD has also been reported to stimulate EGF receptor expression and proliferation in the embryonic palate and ureter epithelial cells, which may be unique to early development (Abbott and Birnbaum, 1989 and 1990a). Thus, data suggest that the effect of BaP or TCDD on EGF receptor mRNA levels may be a species and/or tissue-specific response.

In conclusion, there is no evidence of a direct causal relationship between CYP1A1 induction and EGF receptor downmodulation in choriocarcinoma cells. Metabolism of BaP

by induced CYP1A1 to reactive metabolites or synthesis of a repressor protein may be responsible for the observed loss of EGF receptor in BaP-treated cells.



Cell	Treatment	Bmax [fmole/mg, (%)]	Kd (nM)
BeWo			
	Control	27.7 (100)	0.043
	TCDD, 10 nM	32.7 (118)	0.067
	BaP, 10 μM	18.3 (66)	0.059
JEG-3			
	Control	77.6 (100)	0.048
	TCDD, 10 nM	95.1 (123)	0.063
	BaP, 10 μM	45.1 (58)	0.100

Figure 4-1. Scatchard plot analysis of 125I-EGF binding to control and treated cells. Cells were treated with 0.1% DMSO (control), 10 nM TCDD or 10 μM BaP for 48 hr prior to incubation with increasing concentrations of 125I-EGF (1.25 to 200 pM) at room temperature for 90 min in the presence or absence of excess unlabeled EGF. Specific binding was determined as described under Methods and expressed as fmole 125I-EGF bound per mg total cell protein. B_{max} (fmole/mg) and K_d (nM) values for 125I-EGF binding to BeWo and JEG-3 cells were determined by Scatchard ananlysis of equilibrium binding data.

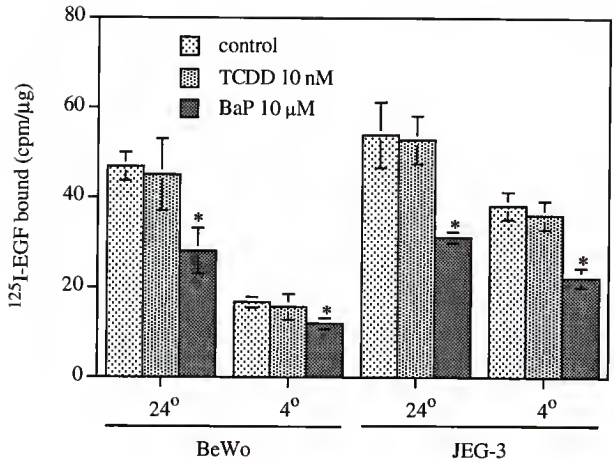


Figure 4-2. Effects of TCDD and BaP on specific binding of ^{125}I-EGF to BeWo and JEG-3 cells. The cells were treated with 0.1% DMSO (control), 10 nM TCDD or 10 μM BaP for 48 hr prior to incubatuion at room temperature (24°C) for 90 min or at 4°C for 5 hr with 100 pM of ^{125}I-EGF in the presence or absence of unlabeled 100 nM EGF. The specific binding was normalized with respect to protein in each sample and expressed as cpm per μg protein of total cell lysate. Values are the mean ± SE of triplicate cultures. * Differs from control at $p < 0.05$ by *t* test.

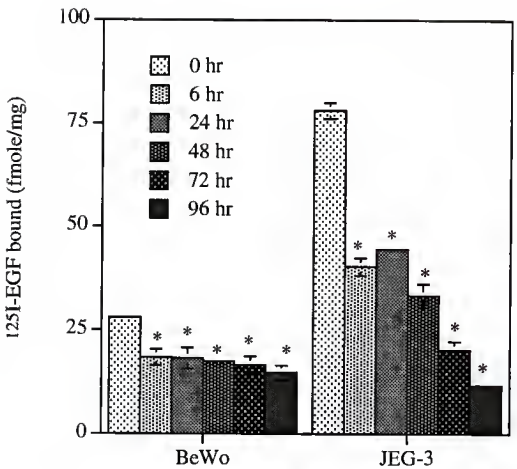


Figure 4-3. Time-course of BaP effects on ^{125}I-EGF binding to whole cells. The BeWo and JGE-3 cells were treated with 10 μM BaP for 6, 24, 48, 72 and 96 hr prior to incubation at room temperature for 90 min with 205 pM ^{125}I-EGF in the presence or absence of unlabeled 328 nM EGF. The specific binding was normalized with respect to protein in each sample and expressed as fmole ^{125}I-EGF bound per mg protein of total cell lysate. Values are the mean ± SE of duplicate cultures. The points without the standard error bars indicate that the individual SEs are too small to be shown. * Differs from time 0 point at $p < 0.05$ by *t* test.

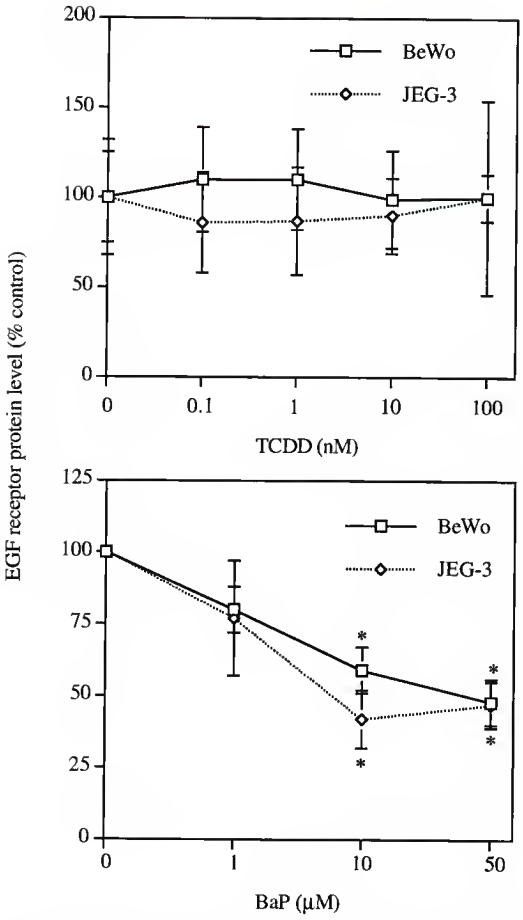


Figure 4-4. Effects of TCDD and BaP on immunoreactive EGF receptor protein in BeWo and JEG-3 cells. The cells were harvested following incubation with TCDD or BaP for 48 hr. Cell protein, 100 μg, was electrophoresed, transferred, and immunostained with sheep anti-EGF receptor. The immunoreactive proteins were then quantitated, with the average value of the controls being arbitrarily set as 100%. Values are the mean ± SE of three separate experiments. *Differs from control at $p < 0.05$ by Fisher PLSD and t test.

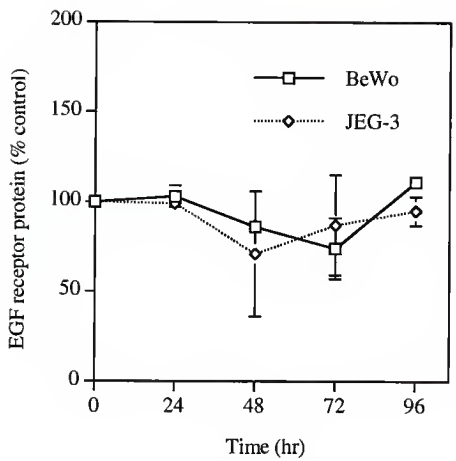


Figure 4-5. Time-course of the effect of TCDD on EGF receptor protein levels. Cells were treated with 10 nM TCDD for various times as indicated. Total cell protein, 100 μg, was separated on 7.5% PAGE, transferred to nitrocellulose, and immunostained with anti-EGF receptor. The immunoreactive band of 170 kDa was quantitated by densitometry scanning, with the average density of the controls being set as 100 %. Values are the mean ± SE of three separate experiments.

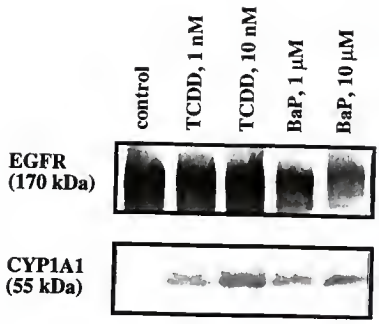


Figure 4-6. Comparison of the effects of TCDD and BaP on EGF receptor and CYP1A1. Cells were treated with TCDD or BaP for 48 hr. Total cell protein, 100 μg, was subjected to PAGE, transferred, and immunostained with anti-EGF receptor or anti-CYP1A1 antibody as described in Materials and Methods. Shown are a representative immunoblot of JEG-3 cells.

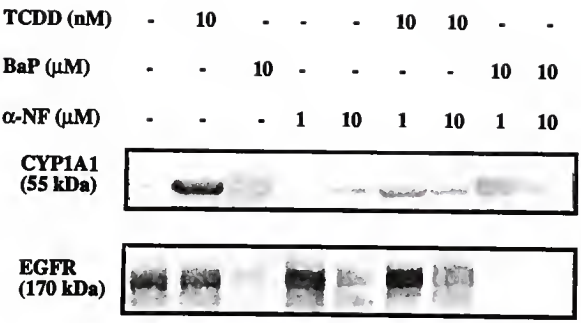


Figure 4-7. Effects of the Ah receptor antagonist α-NF on BaP-mediated changes in EGF receptor expression and CYP1A1 induction in JEG-3 cells. The cells were treated with the respective chemicals at various concentrations for 48 hr. Total cell protein, 100 μg, was separated by PAGE and transferred in duplicate blots. One blot was immunostained with anti-CYP1A1, and the other with anti-EGF receptor.

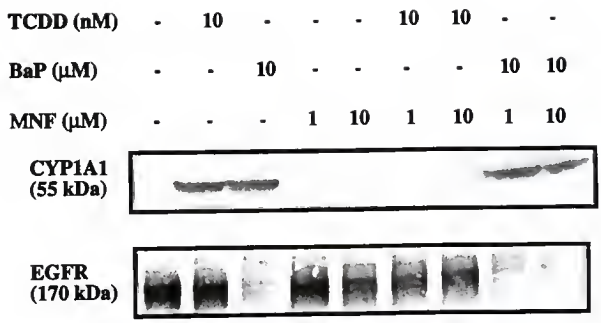


Figure 4-8. Effects of the Ah receptor antagonist MNF on BaP-mediated changes in EGF receptor protein and CYP1A1 induction in JEG-3 cells. The cells were treated with the respective chemicals at various concentrations for 24 hr. Total cell protein, 100 μg, was separated by PAGE and transferred in duplicate blots. One blot was immunostained with anti-CYP1A1, and the other with anti-EGF receptor.

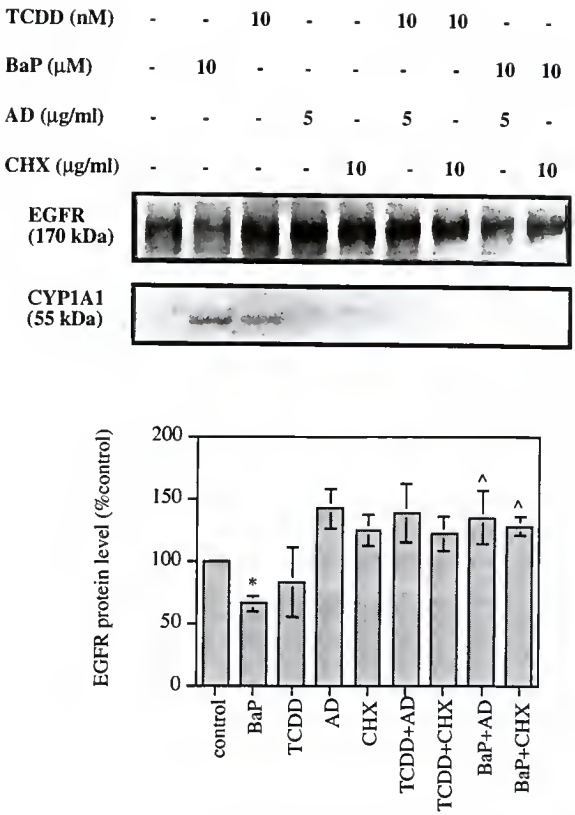


Figure 4-9. Effects of AD and CHX on BaP-mediated changes in EGF receptor protein and CYP1A1 induction in JEG-3 cells. The cells were treated with the respective chemicals at various concentrations for 16 to 24 hr. Total cell protein, 100 μg, was separated by PAGE and transferred in duplicate blots. A) One blot was immunostained with anti-CYP1A1, and the other with anti-EGF receptor. B) Quantitation of the intensity of the 170 kDa EGF receptor band by densitometry, with the control values being set as 100%. Results are the mean ± SE of three separate experiments. * $p < 0.05$ as compared with control; ^ $p < 0.05$ as compared with BaP alone by t test.

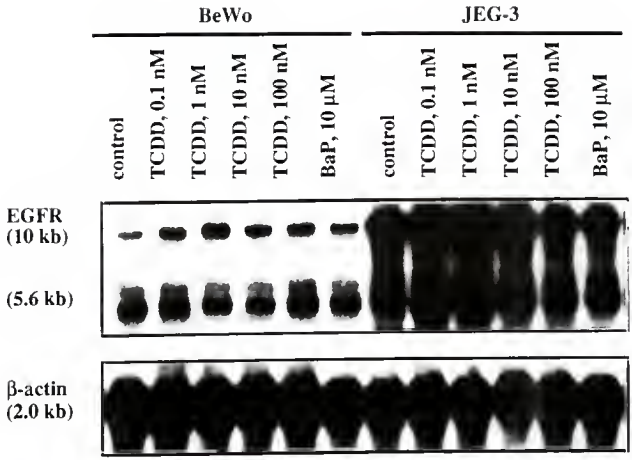


Figure 4-10. Northern blot analysis of EGF receptor mRNA. Cells were incubated with or without TCDD and BaP for 48 hr. Poly(A)$^+$RNA, 10 μg, was denatured, blotted, and hybridized with ^{32}P-labeled cDNA probes as described in Materials and Methods. Shown are representative autoradiograms of the Northern blot.

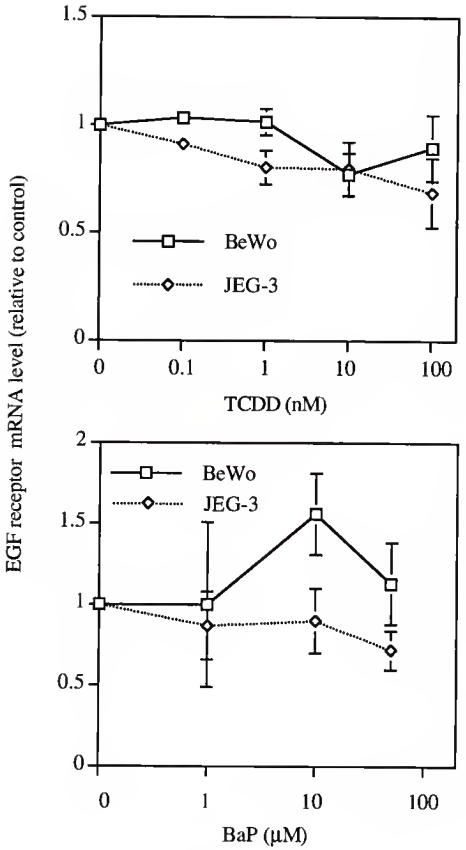


Figure 4-11. Steady state level of EGF receptor mRNA in BeWo and JEG-3 cells following culture in the absence or presence of TCDD and BaP for 48 hr. Poly(A)+RNA, 10 μg (TCDD) or total RNA, 40 μg (BaP), was denatured, blotted, hybridized with the ^{32}P-labeled EGF receptor cDNA probe, and subsequently rehybridized with the ^{32}P-labeled β-actin cDNA as shown in Figure 4-10. Shown are the results from quantitation of the 10 and 5.6 kb transcripts of EGF receptor mRNA, with the ratio of EGF receptor message to β-actin message in the control cells being set as 1. Values are the mean ± SE of three (BaP) or five (TCDD) separate experiments.

CHAPTER 5
EFFECTS OF TCDD AND BAP ON TGF-α, TGF-β1, C-MYC AND PAI-2 GENE EXPRESSION

Introduction

Increasing evidence indicates that TCDD and related Ah receptor ligands act as endocrine disruptors and growth modulators by persistently altering the expression of important growth control genes through sustained activation of the Ah receptor system (as reviewed by Huff et al., 1994; Safe, 1995b; Birnbaum, 1995; DeVito and Birnbaum, 1995) . TCDD has been shown to alter the expression of a number of genes important in cell growth and differentiation, including transforming growth factor (TGF)-α, TGF-β, and plasminogen activator inhibitor 2 (PAI-2) (Abbott and Birnbaum, 1990b; Choi et al., 1991; Sutter et al., 1991; Gaido et al., 1992; Gaido and Maness, 1994; Vogel and Abel, 1995). One of the hallmarks of the effects of TCDD on growth control genes is significant tissue- and species-specificity, as stated earlier in Chapter 1. Little is known regarding the nature of Ah receptor regulated genes in human placental cells.

As discussed in the Introduction Chapter, TGF-α, TGF-β1, c-myc and PAI-2 are all expressed in human placental trophoblast cells and are major partners in the autocrine and paracrine networks which control trophoblast proliferation, differentiation and invasiveness (Goustin et al., 1985; Maruo and Mochizuki, 1987; Feinberg et al., 1989; Ohlsson, 1989; Kauma et al., 1990; Lala and Graham, 1990; Graham et al., 1992; Filla et al., 1993; Horowitz et al., 1993; Lysiak et al., 1993, 1994 and 1995; Hofmann et al., 1994). This study was undertaken to investigate whether TCDD and BaP alter placental function by disrupting the local autocrine and paracrine networks of these important

trophoblast growth control genes. The objective of the study was to identify potential biomarkers of placental toxicity for exposure to environmental chemicals such as TCDD and BaP. The results indicate that the steady state level of TGF-α mRNA was increased by TCDD in BeWo cells, whereas BaP modulated TGF-β1 and c-myc mRNA and protein expression in JEG-3 cells. Evidence further suggests that developmental windows may exist in placental and trophoblast growth for altered responses to environmental chemicals.

Results

Effects of TCDD and BaP on the Steady State mRNA Levels for TGF-α, TGF-β1, PAI-2 and c-myc

Our initial experiments examined the effects of TCDD and BaP on the steady state mRNA levels for TGF-α, TGF-β1, PAI-2 and c-myc in both BeWo and JEG-3 cells (Figure 5-1). Expression of CYP1A1, a gene under direct transcriptional control by the Ah receptor ligands, was used as a positive control. CYP1A1, TGF-β1, PAI-2 and c-myc mRNAs were expressed in choriocarcinoma cells as 3.0, 2.5, 2.3 and 2.4 kb transcripts, respectively, values which are in agreement with previous reports in other tissues (Ohlsson, 1989; Sutter et al., 1991; Greenberg and Ziff, 1984; Gaido et al., 1992). In contrast, the TGF-α mRNA in BeWo and JEG-3 cells was detected as a single 2.3 kb transcript, which is an alternate of the 4.5 kb transcript reported to be expressed by most cells (Lee et al., 1995). CYP1A1 mRNA expression was induced by TCDD at 0.1, 1, 10 and 100 nM in a concentration-dependent manner in both cell lines. Comparable induction of the CYP1A1 mRNA was oberved following treatment with 10 μM BaP.

Exposure to TCDD for 48 hr resulted in a significant concentration-dependent increase in the steady state level of TGF-α mRNA in BeWo cells, but not in JEG-3 cells. In contrast, the steady state mRNA levels for TGF-β1, PAI-2 and c-myc were not altered by TCDD in either cell line (Figure 5-2). Conversely, exposure to 10 μM BaP for 48 hr

significantly increased the steady state TGF-β1 mRNA level in both cell lines, and decreased the steady state c-myc mRNA level in JEG-3 cells, but not in BeWo cells. The steady state levels for TGF-α and PAI-2 mRNA were not altered by BaP in either cell line. The effects of BaP on the steady state TGF-β1 and c-myc mRNA levels were further found to be concentration-related (Figure 5-3). The steady state TGF-β1 mRNA levels were increased 1.5-, 3.0- and 2.9-fold over control, while c-myc mRNA levels were decreased by 38, 61 and 67%, respectively, following exposure of JEG cells to 1, 10 and 20 μM BaP for 48 hr.

We next examined the time course for changes in mRNA expression of TGF-β1 and c-myc. Exposure to 10 nM TCDD for 6 to 120 hr (5 days) had no effect on the steady state levels of TGF-β1 and c-myc mRNA. In contrast, BaP exposure caused a time-dependent change in the steady state TGF-β1 and c-myc mRNA level, and the time dependence was different from that for the induction of CYP1A1 (Figure 5-4). A marked increase in CYP1A1 mRNA level was detected at 6 hr, the earliest time point examined, following exposure to 10 nM TCDD and 10 μM BaP, and levels progressively increased until 72 hr and remained elevated for 5 days, the last time point examined. Significant increases in TGF-β1 mRNA level, however, were not detectable until 24 hr after addition of BaP. By 72 hr, TGF-β1 mRNA level in BaP-treated cells was increased 4-fold over control and remained significantly elevated for 5 days. In contrast, c-myc mRNA level was not changed at 6 and 24 hr, but was significantly decreased by 50% at 72 hr, and further depressed by 60% at 5 days in cultures treated with 10 μM BaP.

Effects of TCDD and BaP on the Rate of TGF-β1 and c-myc Gene Transcription

Nuclear run-off analysis was performed to determine whether the observed changes in the steady state mRNA levels of TGF-β1 and c-myc occurred at the level of mRNA transcription (Figure 5-5). CYP1A1 was used as a positive control for transcriptional activation by TCDD and BaP. Transcription of CYP1A1 was activated at both 6 and 24 hr

following exposure to 10 nM TCDD and 10 μM BaP. Consistent with previous reports (Israel et al., 1985), CHX alone resulted in little increase in the rate of CYP1A1 transcription. However, cotreatment with CHX plus TCDD caused a 3-fold increase in the rate of CYP1A1 transcription compared to TCDD alone. Cotreatment with CHX plus BaP also caused a 2-fold increase in CYP1A1 gene transcription compared to BaP alone. The rate of TGF-β1 transcription was unaltered at 6 hr, but was significantly induced 4-fold in BaP-treated cells at 24 hr. The rate of c-myc transcription was also unaltered at 6 hr, but appeared to be increased at 24 hr following BaP treatment. No significant effect of TCDD treatment was observed on the rate of TGF-β1 and c-myc transcription at either 6 or 24 hr (Figure 5-5). In addition, CHX alone or in combination with TCDD or BaP did not affect the rate of TGF-β1 and c-myc transcription at 6 hr.

Effects of BaP on the Stability of TGF-β1 and c-myc mRNA

Studies were further performed to evaluate the effects of BaP treatment on the stability of TGF-β1 and c-myc mRNA. The decay rate of c-myc mRNA in the presence of actinomycin D was found to be significantly increased in BaP-treated cells (Figure 5-6). The half life of the c-myc mRNA was shortened from 30 min in control cells to 18 min in BaP-treated cells. Thus results indicate that the decrease in the steady state c-myc mRNA level following BaP treatment could be accounted for by the decrease in stability of the c-myc mRNA. In contrast, no alteration in TGF-β1 mRNA stability was observed. The half life of the TGF-β1 mRNA was approximately 10 hr in both control and BaP-treated cells. The finding that the stability of TGF-β1 mRNA was not increased following BaP exposure further supports the previous finding that the increase in the steady state TGF-β1 mRNA level is due to the increase in the rate of TGF-β1 transcription.

Effects of TCDD and BaP on TGF-β1 and c-Myc Protein Levels

TGF-β1 was undetectable in the media from control or treated JEG-3 cells using an ELISA assay which detects biologically active TGF-β1. Activation of the conditioned media (CM) by low pH, however, converts latent TGF-β1 to the bioactive form such that a TGF-β1 concentration of 1280 ± 165 pg/ml was measured in the CM of control cells. Data in Figure 5-7 show that BaP treatment for 48 hr increased TGF-β1 secretion by JEG-3 cells in a concentration-related manner. Due to the high variability between assays, however, the increase was not statistically significant. The secretion of TGF-β1 also appeared to be increased slightly following exposure to TCDD for 48 hr.

Western blot analysis using an antibody to human c-myc protein identified a minor and a major immunoreactive band with an apparent molecular mass of approximately 67 (Myc-1) and 64 (Myc-2) kDa (Figure 5-8), respectively. These observed values for Myc-1 and -2 in choriocarcinoma cells are in agreement with previous reports in other tissues (Munger et al., 1992; Packham and Cleveland, 1995). Quantitation of the major 64 kDa band revealed a concentration-dependent decrease in Myc level following BaP, but not TCDD, treatment. Myc levels were significantly decreased by 38, 46, 54 and 50% at 1, 5, 10 and 20 μM BaP, respectively, after treatment for 48 hr. Quantitation of the minor 67 kDa band showed similar results (data not shown).

Discussion

TCDD modulation of growth control genes shows significant tissue and species specificity (Safe, 1995b; Birbaum, 1995). Our studies indicate that TCDD increased TGF-α mRNA level in BeWo but not in JEG-3 human placental cells (Figure 5-1 and 2). The differential response to TCDD observed in BeWo cells compared with JEG-3 cells may reflect developmental differences in the state of differentiation of these two cell lines, the latter being more invasive into cultured reepithelialized endometrial fragments (Grümmer et

al., 1994). In this regard, TGF-α mRNA level has been shown to be increased by TCDD in human primary and SCC-12F keratinocyte and breast cancer MCF-7 cells (Gaido et al., 1992; Choi et al., 1991; Vogel and Abel, 1995), but not in mouse and rat liver (Lin et al., 1991; Vanden Heuvel et al., 1994). Consistent with the earlier observations in human keratinocyte SCC-12F and breast cancer MCF-7 cell lines (Gaido et al., 1992; Vogel and Abel, 1995), the present study has shown that the steady state mRNA level or transcription rate of TGF-β1 was not altered by TCDD in either BeWo or JEG-3 cell line (Figure 5-1, 2, 4 and 5). It warrants note, however, that TCDD has been shown to reduce the expression of TGF-α and TGF-β1 in mouse embryonic palate epithelial and mesenchymal cells (Abbott and Bimbaum, 1990), which may be unique to early development.

In choriocarcinoma cells, the steady state c-myc mRNA level was found to be unchanged following TCDD exposure, which is in agreement with an earlier report in mouse Hepa-1 hepatoma cells (Puga et al., 1992). TCDD has been shown to decrease c-Myc DNA binding activity by modulating its state of phosphorylation in guinea pig adipose tissue (Enan and Matsumura, 1994 and 1995). It has been further reported that the expression of PAI-2 mRNA was increased by TCDD in human SCC-12F keratinocyte, primary hepatocyte, monocytic U937, hepatoma HepG2 and breast cancer MDA-MB 231 cells (Sutter et al., 1991; Dohr et al., 1995; Gohl et al., 1996). However, Vanden Heuvel et al. (1994) found that the PAI-2 mRNA level was not altered by TCDD in rat liver, which is in agreement with our present finding in choriocarcinoma cells.

Regardless of the large variability in TCDD responsiveness, CYP1A1 mRNA is uniformly induced by TCDD in human keratinocytes, hepatocytes, HepG2 and breast cancer cells, mouse and rat liver (Lin et al., 1991; Sutter et al., 1991; Gaido et al., 1992; Choi et al., 1991; Vanden Heuvel et al., 1994; Dohr et al., 1995; Vogel and Abel, 1995; Gohl et al., 1996), as well as in choriocarcinoma cells as shown by this study (Figure 5-1 and 4). Our data, therefore, provide further support for action by TCDD through the

classic Ah receptor mechanism with tissue- and gene-specific differences in responsiveness.

The mechanisms responsible for the tissue- and gene-specific differences in TCDD responsiveness, however, are poorly understood. Gradin et al. (1993) found that the nonresponsiveness of normal human fibroblasts to TCDD was due to the presence of a constitutive XRE-binding factor. In a study of the relationships between DNA sequence, receptor binding, and TCDD responsiveness, Lusska et al. (1993) demonstrated that the Ah receptor-DNA binding event *per se* was not sufficient to confer TCDD responsiveness upon a linked gene. Based upon a comparison of the DNA sequences of the Ah receptor binding sites, the authors suggested a "functional consensus" recognition sequence, which is more extended in length than the "core"-binding sequence 5'-TNGCGTG-3'. These authors further proposed that the liganded Ah receptor can function in either positive or negative fashion, depending on the regulatory context. Thus, the action of some cell- or gene-specific factors might account for some of the gene-to-gene differences in TCDD responsiveness.

BaP is a transplacental carcinogen (Bulay and Wattenberg, 1970), and its mutagenic effects are well characterized (Levin et al., 1978). However, the biochemical events associated with BaP exposure other than covalent DNA binding are poorly understood. This study demonstrated for the first time that exposure of JEG-3 cells to BaP caused a persistent increase in TGF-β1 mRNA and a sustained depression in c-myc mRNA and protein levels, along with concomitant induction of CYP1A1 mRNA (Figure 5-3, 4 and 8). Conversely, BaP was reported to increase the steady state c-myc mRNA level in rat aortic smooth muscle cells (Sadhu et al., 1993). In addition, our data in Chapter 3 and 4 have demonstrated that EGF receptor protein level was significantly decreased, accompanying induction of CYP1A1 protein following BaP exposure. Thus, growing evidence indicates that, like TCDD, BaP also leads to species-, tissue- or gene-specific modulation of growth control gene expression.

Our data indicate that BaP-induced changes in the steady state TGF-β1 and c-myc mRNA level may not be a primary response to the interaction of the liganded Ah receptor complex with specific xenobiotic responsive elements (XREs) in the promoter region. First, there is a temporal lag in the effect of BaP on TGF-β1 and c-myc gene expression relative to CYP1A1 induction (Figure 5-4 and 5). Second, TCDD has no effect on these two genes in JEG-3 cells, despite the CYP1A1 induction (Figure 5-1, 2, 4 and 5). Transcriptional activation of CYP1A1 is a well-characterized primary response to the binding of the ligand-bound Ah receptor complex to upstream XREs of the CYP1A1 transcription start site (Hankinson, 1995) and was observed at 6 hr, the earliest time point examined, in JEG-3 cells following treatment with both TCDD and BaP (Figure 5-5). However, the rate of TGF-β1 transcription was not altered by BaP at 6 hr, nor by TCDD, in contrast to the early increase in CYP1A1 transcription. Therefore, although the BaP-mediated increase in the steady state TGF-β1 mRNA level involves direct transcriptional regulation of the TGF-β1 gene, this may not necessarily be regulated directly by the liganded Ah receptor complex, but more likely represents a secondary response. In this regard, MacLeod et al (1995) recently demonstrated that BPDE modification of GC-box sequences in the promoter region of the hamster adenosine phosphoribosyl transferase gene caused a substantial increase in the apparent affinity for the transcription factor Sp1 (MacLeod et al., 1995). Moreover, this study has shown that Sp1 bound to the BPDE-modified non-GC-box DNA fragment with relatively high affinity. This type of evidence suggests that BPDE-DNA adduct sites can interact with Sp1, which may selectively affect transcription of specific genes.

TGF-β has been reported to downmodulate c-myc mRNA expression in the mouse BALB/MK keratinocyte cell line, secondary cultures of human keratinocytes, and the human MOSER colon carcinoma cell line (Pietenpol et al., 1990; Munger et al., 1992; Mulder et al., 1988). The block in c-myc expression by TGF-β1 has further been shown through inhibition of transcriptional initiation (Pietenpol et al., 1990). In the present study,

the time course of upmodulation of TGF-β1 by BaP preceded the downmodulation of c-myc expression (Figure 5-4); however, it remains to be determined whether there is any causal relationship for the inverse changes between these two factors. In JEG-3 cells, the observed decrease in the steady state c-myc mRNA level following BaP treatment appears to occur posttranscriptionally, based upon evidence from the mRNA stabilization assay which showed decreased stability of c-myc mRNA following BaP treatment (Figure 5-6). Altogether, the present study demonstrates that regulation of gene expression by BaP results in differential changes in mRNA levels for specific genes and can occur by multiple mechanisms, including transcriptional and posttranscriptional regulation, as previously described for TCDD (Gaido et al., 1992; Hankinson, 1995).

TGF-α has been shown to stimulate trophoblast proliferation (Lysiak et al., 1993) and 17β-hydroxysteroid dehydrogenase type 1 activity which catalyzes the reversible interconversion of estrone and estradiol in placental cells (Lewintre et al., 1994). Thus, our finding that TCDD increased the TGF-α mRNA level in BeWo but not in JEG-3 cells implicates that TCDD may interfere with normal human trophoblast proliferation and endocrine function at a certain stage of placental development.

In this study, changes in c-Myc protein and TGF-β1 levels were observed which correlated with the alterations in c-myc and TGF-β1 mRNA levels following exposure of JEG-3 cells to BaP, which may be relevant to retardation of fetal growth. These two genes have been reported to be regulated in concert with changes that affect placental cell proliferation, differentiation and invasiveness, with TGF-β1 being anti-proliferative and anti-invasive, and c-myc being linked with proliferative and invasive trophoblast activities (Goustin et al., 1985; Ohlsson, 1989; Schmid et al., 1989; Lala and Graham, 1990; Graham and Lala, 1991; Graham et al., 1992; Cross et al., 1994). Studies with human trophoblasts have shown that TGF-β1 upregulates tissue inhibitor of metalloproteinases and extracellular matrix proteins such as oncofetal fibronectin, as well as downregulates the activity of u-PA and collagenase type IV (Lala and Graham, 1990; Graham and Lala, 1991;

Graham et al., 1994; Guller et al., 1995). Interestingly, c-Myc has been found to repress collagen gene expression (Packham and Cleveland, 1995). Therefore, BaP-mediated upmodulation of TGF-β1 and downmodulation of c-myc may lead to accumulation of extracellular matrix such as collagen in human placenta. Indeed, placentas from women who smoke cigarettes have been shown to exhibit thickening of the basement membrane and increased collagen content of the villous stroma (Asmussen, 1980).

Upmodulation of TGF-β1 may also disrupt placental endocrine function. TGF-β1 has been found to inhibit both basal and EGF-stimulated human chorionic gonadotropin and placental lactogen secretion by primary cultures of cytotrophoblasts (Morrish et al., 1991) and mouse growth hormone releasing factor secretion by placenta (Yamaguchi et al., 1994). In fact, serum levels of human placental lactogen were found to be lower in heavy smokers than those in nonsmokers (Mochizuki et al., 1984). In mice c-myc mutant embryos are small and retarded in development compared with their littermates (Davis et al., 1993), providing direct evidence that c-Myc is necessary for normal embryonic development. Therefore, the observed alterations in TGF-β1 and c-myc gene expression may underlie mechanisms by which xenobiotics such as those found in cigarette smoke cause fetal intrauterine growth retardation.

In summary, the present study has demonstrated that 1) TCDD increased TGF-α mRNA expression in BeWo but not in JEG-3 cells; 2) TCDD had no effect on the steady state mRNA levels for TGF-β1, PAI-2 and c-myc in either cell line; 3) BaP increased TGF-β1 mRNA and protein expression at the level of gene transcription, while c-myc mRNA and protein levels were decreased via a posttranscriptional destabilization of the mRNA in JEG-3 cells; 4) BaP also caused a slight increase in TGF-β1 mRNA level in BeWo cells; 5) BaP had no effect on TGF-α and PAI-2 mRNA level in either cell line. The significance of these changes is that a specific temporal expression of TGF-α, TGF-β1 and c-myc is important for the control of trophoblast proliferation, differentiation and invasiveness. The disruption of coordinated TGF-α, TGF-β1 and c-myc gene expression may directly

interfere with normal placental development, which subsequently may lead to altered fetal growth. In addition, these data imply that different mechanisms may be involved in the placental toxicity of TCDD and BaP.

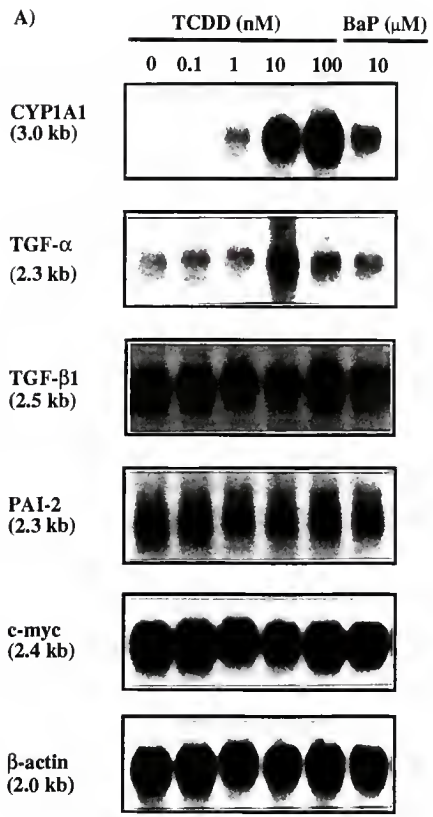


Figure 5-1. Northern blot analysis following treatment of cells with DMSO (control), TCDD or BaP for 48 hr. A) BeWo; B) JEG-3. Poly(A)+ RNA, 10 μg, was separated on 1.0% formaldehyde-agarose gel, transferred to nylon membrane, and probed with ^{32}P-labeled cDNAs as indicated.

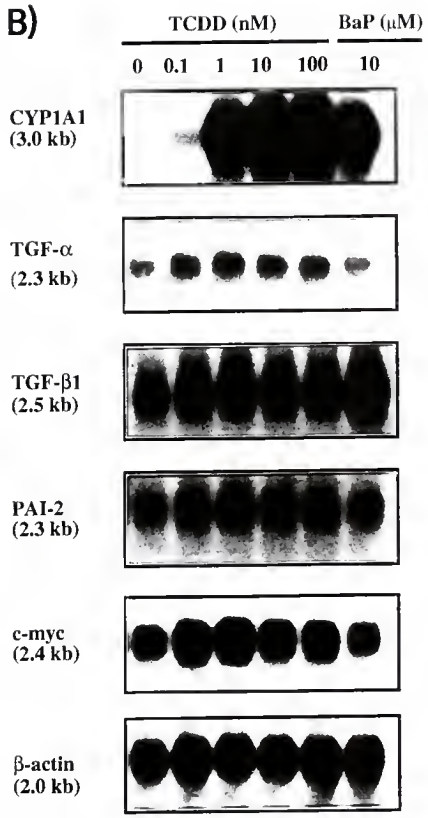


Figure 5-1 continued

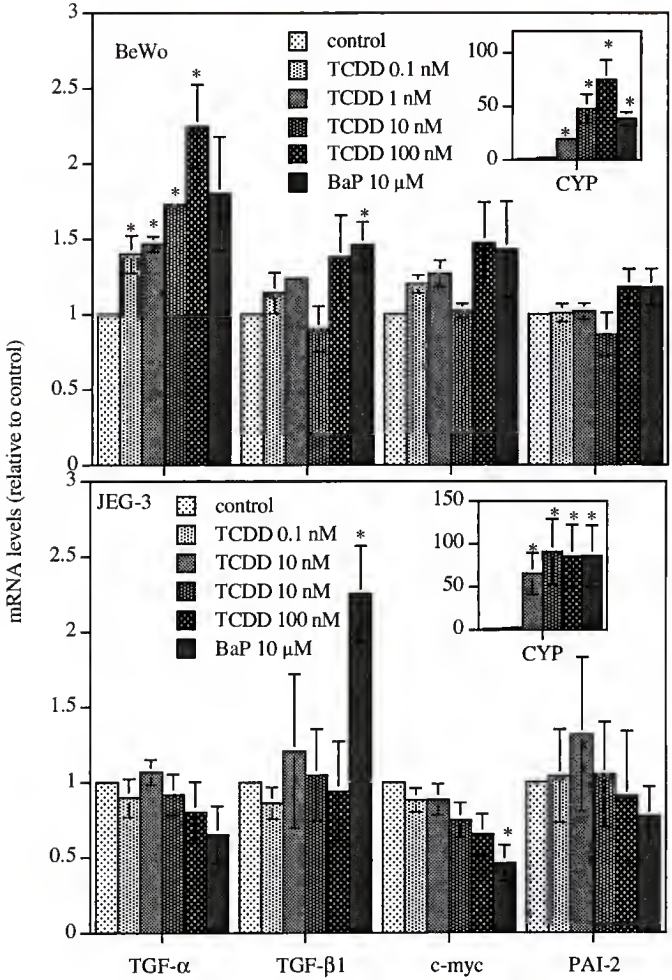


Figure 5-2. Effects of TCDD on the steady state mRNA levels of TGF-α, TGF-β1, c-myc and PAI-2. Each hybridization band as shown in Figure 5-1 was quantitated by densitometry and normalized to β-actin message, with the control values being set as 1. Values are the mean ± SE of three experiments. The inserts shown are for CYP1A1 induction as a positive control. *Differs from control by Fisher PLSD or *t* test.

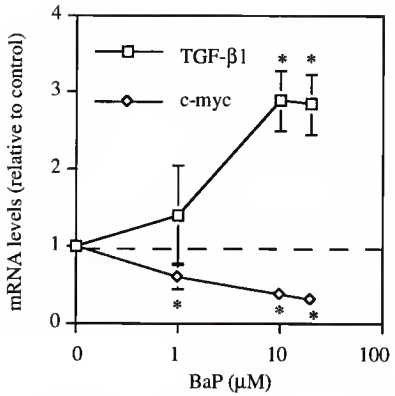


Figure 5-3. Concentration-dependent changes in the steady state mRNA levels for TGF-β1 and c-myc following BaP treatment. JEG-3 cells were treated with 0.1% DMSO or 1, 10 and 20 μM BaP in 0.1% DMSO for 48 hr and poly(A)$^+$ RNA was isolated for Northern analysis. The blots were sequentially hybridized with the ^{32}P-labeled TGF-β1, c-myc and β-actin probes, and quantitated by densitometry, with the ratio of each message to β-actin message in the control cells being set as 1. Values are the mean ± SE of four separate experiments. The points without the standard error bars indicate that the individual SEs are too small to be shown. *$p < 0.05$ as compared with control by PLSD and *t* test.

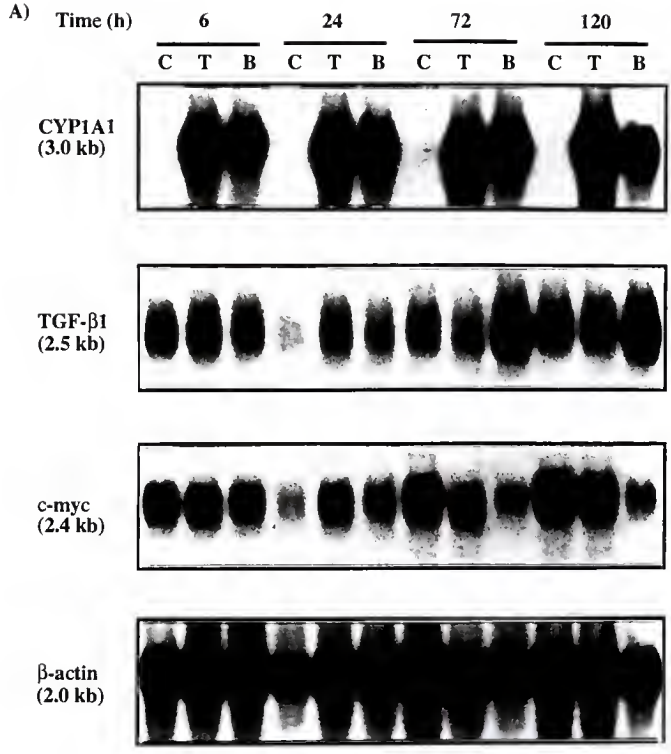


Figure 5-4. Time-dependent changes in the steady state mRNA levels of TGF-β1 and c-myc. JEG-3 cells were treated with 0.1% DMSO (C), 10 nM TCDD (T), or 10 μM BaP (B). Poly(A)$^+$ RNA was extracted for Northern analysis at 6, 24, 72, and 120 hr after treatment. The blots were hybridized with ^{32}P-labeled probes as indicated. A) Autoradiograms of the Northern blot; B) Quantitation of the TGF-β1 and c-myc mRNA, with the ratio of each message to β-actin message in the control cells being set as 1. Values are the mean ± SE of three experiments. The points without the standard error bars indicate that the individual SEs are too small to be shown. $*p < 0.05$ as compared with control by *t* test.

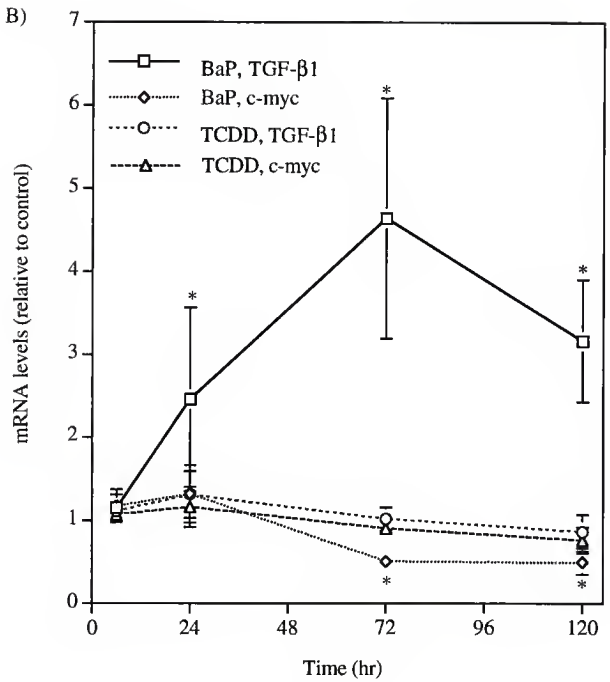
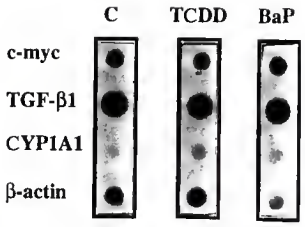


Figure 5-4 continued

A)



B)

Treatment	TGF-β1	c-myc	CYP1A1
6 h			
TCDD, 10 nM	1.01 ± 0.12	0.99 ± 0.31	2.89 ± 2.2.03
BaP, 10 μM	1.03 ± 0.19	1.07 ± 0.33	2.29 ± 1.52
CHX, 10 μg/ml	1.25 ± 0.37	1.02 ± 0.21	0.99 ± 0.20
TCDD + CHX	1.06 ± 0.20	0.97 ± 0.21	7.96 ± 1.6*
BaP + CHX	1.14 ± 0.47	0.86 ± 0.16	4.45 ± 1.42*
24 h			
TCDD, 10 nM	1.00 ± 0.15	0.73 ± 0.16	18.83 ± 17.45
BaP, 10 μM	4.14 ± 1.23*	3.99 ± 1.57	12.80 ± 2.49*

Figure 5-5. Effects of TCDD and BaP on the rate of TGF-β1 and c-myc transcription by nuclear run-off assay. Cells were treated with 10 nM TCDD, 10 μM BaP or 0.1% DMSO (C) in the presence or absence of 10 μg/ml CHX. Nuclei were isolated at 6 and 24 hr and nuclear run-off assay was performed. The [α-^{32}P] UTP labeled nascent transcripts were hybridized with nylon blots with 2 μg of specific c-myc, TGF-β1, CYP1A1 and β-actin cDNA in each dot. A) A representative autoradiogram of the dot blots following 24 hr treatment; B) Hybridization signal was quantitated by densitometry and normalized to β-actin. Results are expressed as the relative level of gene transcription in treated cells *versus* control cultures. Values are the mean ± SE of two (TCDD) to three (BaP) experiments. *$p < 0.05$ as compared with control by *t* test.

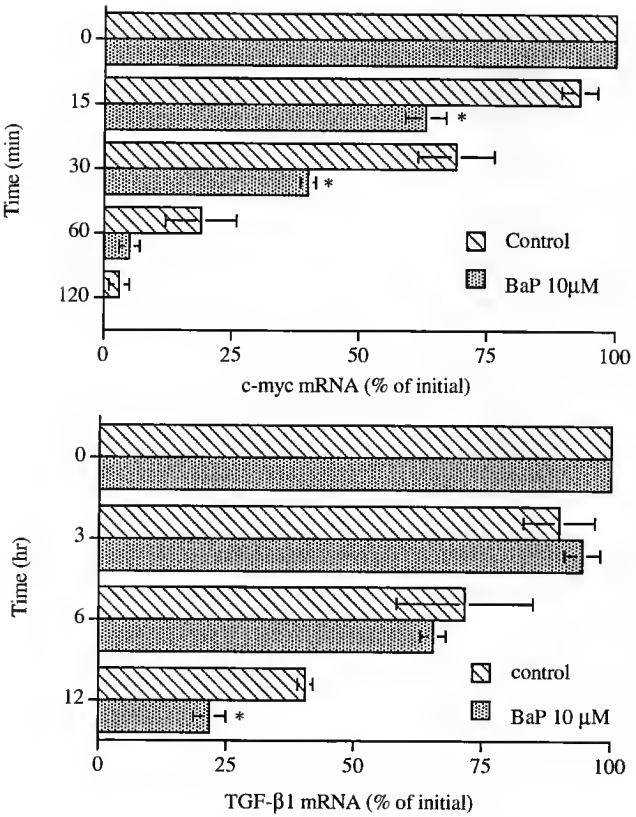


Figure 5-6. Effects of BaP on the stability of c-myc and TGF-β1 mRNA in the presence of the RNA synthesis inhibitor AD. JEG-3 cells were pretreated with 0.1% DMSO or 10 μM BaP for 24 hr, and AD was added directly to the media to a final concentration of 5 μg/ml. Total RNA was extracted for Northern analysis at the different time points after the addition of AD as indicated. The hybridization signal was quantitated, with the ratio of each message to β-actin message at 0 time point being set as 100%. $*p < 0.05$ as compared with control by *t* test.

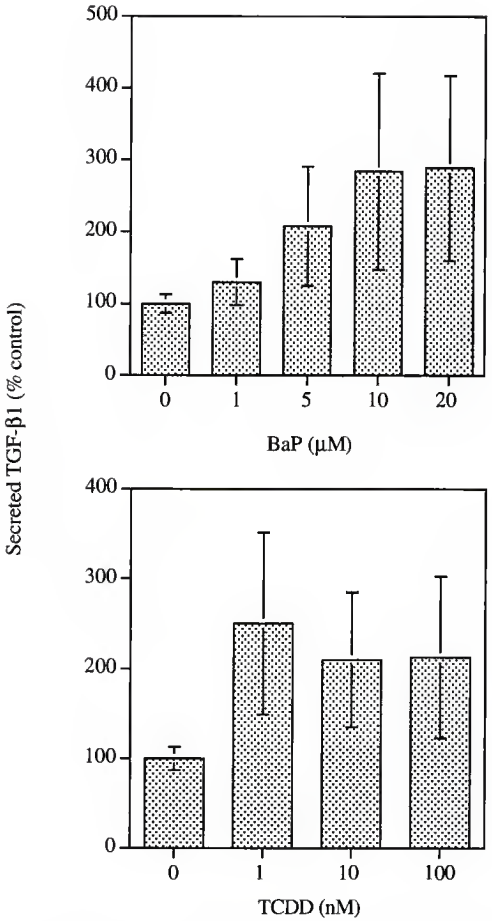
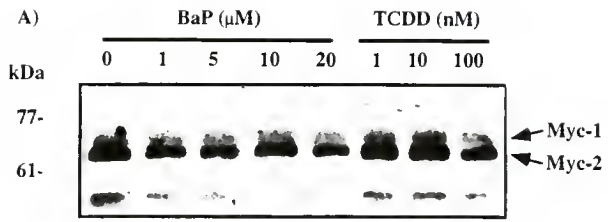


Figure 5-7. Effects of BaP and TCDD on the secretion of TGF-β1 by JEG-3 cells. Cells were treated with BaP or TCDD for 48 hr, and the conditioned medium was then collected and acid-activated for assay of TGF-β1 levels. Values are the mean ± SE of seven experiments.



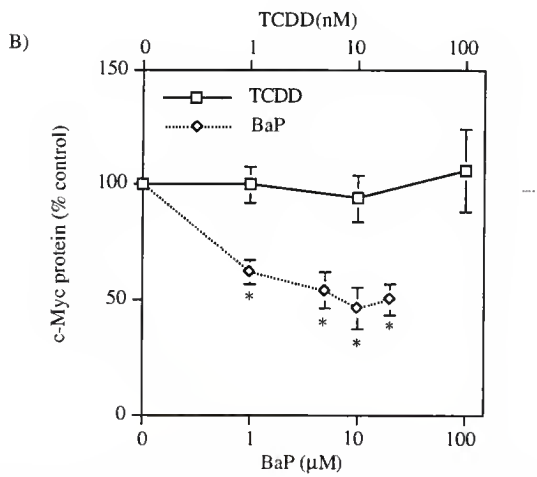


Figure 5-8. Effects of TCDD and BaP on c-Myc protein expression in JEG-3 cells. Cells were treated with the indicated concentrations of BaP or TCDD for 48 hr. Total cell lysate (100 μg) was separated on 10% PAGE, transferred to nitrocellulose and immunostained with anti-human c-myc. The bands were visualized by ECL detection (A), and the intensity of the 64 kDa Myc-2 band were quantitated by densitometry (B). Similar results were obtained with quantitation of the minor 67 kDa band (data not shown). Values are the mean ± SE of seven experiments. * $p < 0.05$ as compared with control by Fisher PLSD.

CHAPTER 6
EVALUATION OF THE EFFECTS OF TCDD AND BAP ON CELLULAR GROWTH AND ENDOCRINE RESPONSES OF BEWO AND JEG-3 CELLS

Introduction

The main objective of this project is to identify the target genes that represent primary biologic responses to the environmental chemicals TCDD and BaP. The challenge in this study is to use the knowledge of the alterations induced in gene expression by TCDD and BaP to further test the hypothesis regarding likely changes in the more complex biologic endpoints of cell proliferation, migration and endocrine function. This study has demonstrated that 1) TCDD increases TGF-α mRNA expression in BeWo cells, 2) BaP decreases EGF receptor levels in both BeWo and JEG-3 cells, 3) BaP increases TGF-β1 mRNA expression in both cell lines, 4) BaP decreases c-myc mRNA and protein expression in JEG-3 cells. The next question was whether these changes in trophoblast growth control networks alter characteristic trophoblastic cell functions of proliferation, hormone secretion and invasiveness.

Results

Effects of TCDD and BaP on Cell Proliferation as Measured by the MTT Conversion Assay

The MTT conversion assay was used to determine whether TCDD and BaP treatment was associated with cytotoxic effects on cell division. Data in Figure 6-1 show that exposure to 0.1-100 nM TCDD or 1 nM-50 μM BaP had no significant effect on cell proliferation up to 48 hr in BeWo and JEG-3 cells cultured in the presence of serum. In

contrast, under serum-free conditions, BeWo and JEG-3 cell proliferation was inhibited 30-50% with TCDD at 10 and 100 nM and BaP at 10 and 50 μM. Thus TCDD and BaP exposure for 48 hr at higher concentrations adversely affected cell viability only under serum-free conditions.

We next examined whether the BaP-mediated loss of EGF receptors altered EGF-stimulated cell proliferation. EGF at concentrations of 100 (16 nM) and 200 ng/ml significantly stimulated cell proliferation 1.5 to 2-fold under serum-free conditions in both cell lines (Figure 6-2). When cells were exposed to EGF (100 ng/ml) and BaP together for 48 hr, the EGF stimulation of cell proliferation was still observed at 10 μM BaP, but was significantly decreased from control at 50 μM BaP in both cell lines. A two factor ANOVA, however, indicates that there was no significant interactive effect between BaP and EGF on BeWo cell proliferation. In contrast, there was a significant interactive effect of BaP and EGF on JEG-3 cell proliferation ($p < 0.05$). In this regard, a higher concentration of EGF (200 ng/ml) protected JEG-3 cells, but not BeWo cells, from the loss of EGF stimulation of cell proliferation in the presence of 50 μM BaP. This protective effect of the higher dose of EGF in JEG-3 cells is similar to the protective effect of serum on maintaining cell viability in the presence of 50 μM BaP (Figure 6-1).

Effects of TCDD and BaP on JEG-3 Cell Proliferation as Measured by [^{3}H]Thymidine Incorporation and Cell Number Changes

We next examined whether longer exposure to BaP and TCDD would alter JEG-3 cell proliferation in the presence of serum using the [^{3}H]thymidine incorporation assay. A time-dependent inhibition of cell proliferation was observed following BaP exposure over a 7 day period (Figure 6-3). The [^{3}H] incorporation was significantly decreased by 42, 86 and 81% at 3, 5 and 7 days, respectively, following 10 μM BaP treatment in the presence of serum. Data in Figure 6-4 show that the inhibitory effect was also concentration-dependent following exposure to BaP for 5 days. [^{3}H] Thymidine incorporation was

significantly inhibited by 55, 67, 73, and 76% at 1, 5, 10 and 50 μM BaP, respectively. Similar results were observed in parallel experiments in which cell number was quantitated. In contrast, no alterations in the [^{3}H]thymidine incorporation and cell number was observed following exposure to 1, 10 and 100 nM TCDD for 5 days or to 10 nM TCDD for 1 to 7 days (Figure 6-3 and 4).

Effects of TCDD and BaP on BeWo Cell Proliferation as Measured by [^{3}H]Thymidine Incorporation

We also examined whether longer exposure of TCDD and BaP altered BeWo cell proliferation. No alterations in [^{3}H]thymidine incorporation was observed following exposure to 10 nM TCDD for 3, 5 and 7 days or to 1, 10 and 100 nM TCDD for 5 days (Figure 6-5). In contrast, exposure to 10 μM BaP significantly decreased BeWo cell proliferation by 29, 49 and 46% at 3, 5 and 7 days, respectively. The inhibition was also concentration-dependent. [^{3}H]Thymidine incorporation was significantly decreased by 30, 46, 51 and 54% following exposure for 5 days to 1, 5, 10 and 20 μM BaP, respectively.

Effects of TCDD and BaP on Secretion of the Hormone hCG

The next experiment evaluated whether TCDD and BaP exposure was associated with altered trophoblast endocrine function as measured by secretion of the peptide hormone hCG. Data in Figure 6-6 show that TCDD and BaP treatment for 48 hr significantly inhibited basal hCG secretion by BeWo cells, but not by JEG-3 cells. Hormone secretion by BeWo cells was reduced by 29, 40 and 30% at 1, 10 and 100 nM TCDD after 48 hr treatment, respectively. The hCG concentration in the BeWo cell media was significantly reduced by 41, 56 and 64% at 1, 10 and 50 μM BaP after 48 hr exposure, respectively (Figure 6-6). In JEG-3 cells, however, the level of basal hCG secretion following TCDD or BaP treatment remained at a high level, as in control cells.

EGF is known to be a stimulant of hCG secretion and we next evaluated whether the BaP-mediated loss of EGF receptors was correlated with an alteration in EGF-stimulated hCG secretion. BeWo cells were first incubated with BaP for 48 hr, and then treated with EGF for another 24 hr in serum-free medium. As shown in Figure 6-7, EGF stimulated hCG secretion 3-fold in untreated control BeWo cells; the response, however, was significantly decreased by 29 and 43% in BeWo cells pretreated with BaP at 1 and 10 μM, respectively. In contrast, in JEG-3 cells, BaP pretreatment did not alter the stimulation of hCG secretion by EGF, with EGF at 100 ng/ml producing an appoximate 2-fold increase in all three groups of JEG-3 cells. Thus differential effects of BaP were observed on hCG secretion by BeWo and JEG-3 cells.

Differential Effects of TCDD and BaP on JEG-3 Cell Invasiveness

A final experiment evaluated JEG-3 cell invasiveness using the Boyden Chamber in which cells were plated on Matrigel-coated membranes. Figure 6-8 shows that JEG-3 cell invasion through Matrigel coated filters was significantly inhibited by BaP treatment, whereas TCDD pretreatment significantly stimulated invasion. The number of invasive cells was reduced by 84, 92, and 86% at 1, 10 and 20 μM BaP after 48 hr pretreatment, respectively (Figure 6-9). In contrast TCDD pretreatment for 48 hr increased the number of invasive cells 1.4- and 2.7-fold at 10 and 100 nM, respectively.

Discussion

Trophoblast proliferation has been shown to be negatively regulated by TGF-β1 and positively correlated with the level of c-Myc and EGF receptors (Gross et al., 1994; Lala and Graham, 1990; Ohlsson, 1989). In the present study, the observed inhibition of JEG-3 cell proliferation and invasion by BaP may well be mediated by the upmodulation of TGF-β1 and the downmodulation of c-Myc and EGF receptor following BaP treatment. Our findings may also have direct relevance to earlier observations that cytotrophoblast cell

proliferation was decreased in placentas from smokers (Sachs, 1989; Genbacev et al., 1995). In this study, TCDD was not found to affect EGF receptor, TGF-β1, PAI-2 and c-myc gene expression. Although the steady state TGF-α mRNA level was increased by TCDD in BeWo cells, our effort to quantitate TGF-α protein in conditioned medium or total cell lysate by Western analysis was not successful. Data have shown that choriocarcinoma cell proliferation was not altered following TCDD exposure. Most importantly, it was a major unexpected finding that JEG-3 cell invasiveness was significantly increased by TCDD exposure, suggesting that some factors which control trophoblastic cell invasion were altered by TCDD other than EGF receptor, TGF-α, TGF-β1, PAI-2 and c-myc. In this regard, human keratinocytes (Gaido and Maness, 1994) exposed to TCDD were found to have increased expression of the proteinase u-PA, which can bind to the u-PA receptor in human trophoblast cells and lead to local proteolysis (Lala and Graham, 1990; Strickland and Richards, 1992).

Trophoblast invasion through the uterine epithelium is promoted by proteinases that degrade the extracellular matrix (ECM), and, conversely, limited by proteinase inhibitors that broadly or specifically inhibit the proteinase activities (Lala and Graham, 1990; Strickland and Richards, 1992; Cross et al., 1994). In addition, evidence indicates that the trophoblast alters its migratory phenotype during placental development, such that invasiveness decreases as the cytotrophoblast differentiates. The terminally-differentiated syncytiotrophoblast is nonproliferative and noninvasive (Lala and Graham, 1990). Thus our finding that TCDD increased JEG-3 cell invasiveness suggest that the cells may be less differentiated following TCDD exposure.

Trophoblast proliferation and invasion are coordinatedly regulated by local autocrine and paracrine growth control networks. Both proliferation and invasion are downregulated by TGF-β1 that is secreted by the decidua and trophoblast (Graham and Lala, 1991; Graham et al., 1992). However, recent data from Lala's laboratory provide evidence that human trophoblast proliferation and invasion can be independently regulated

by locally produced growth factors. For example, insulin-like growth factor (IGF)-II, produced by the cytotrophoblast, stimulates invasion and migration without affecting proliferation (Lala and Lysiak, 1994; Lysiak et al., 1994a; Irving and Lala, 1995), whereas EGF and TGF-α, secreted by the trophoblast and the decidua, do not influence invasion but upregulate proliferation (Lysiak et al., 1993 and 1994b). These findings may partially explain our observation that TCDD upregulated JEG-3 cell invasion without affecting cell proliferation.

The mitogenic response of choriocarcinoma cells to EGF can be observed under serum-free conditions, which is in agreement with the reported mitogenic action of EGF or TGF-α on cytotrophoblasts (Filla et al., 1993; Maruo et al., 1992). In choriocarcinoma cells, we found that BaP inhibited both basal and EGF-stimulated cell proliferation under serum-free conditions. The inhibitory effect of BaP on EGF-stimulated proliferation in BeWo cells appears not to be specific since the relative percentage decrease in both groups was similar and no significant interaction of BaP and EGF was observed by two-way ANOVA. On the other hand, there was a significant interaction between BaP and EGF on JEG-3 cell proliferation, suggesting that different mechanisms may be involved in the regulation of BeWo and JEG-3 cell proliferation. TCDD has been shown to inhibit MCF-7 human breast cancer cell proliferation (Biegel and Safe, 1994) but to stimulate EGF receptor expression and proliferation in the mouse embryonic palate and ureter epithelial cells (Abbott and Birnbaum, 1989 and 1990a). In BeWo and JEG-3 cells, we found that TCDD did not affect either EGF binding or cell proliferation.

An earlier clinical study found in pregnant women that serum levels of human placental lactogen were lower in cigarette smokers than in nonsmokers (Mochizuki et al., 1984). The present study found that the loss of EGF receptors in choriocarcinoma cells is correlated with decreased basal and EGF-stimulated hCG secretion in BeWo cells, but not in JEG-3 cells. The basal hCG secretion was also found to be decreased by TCDD in BeWo cells, again not in JEG-3 cells. In this regard, recent studies with human

extravillous trophoblasts in explant culture have observed significant gestation-specific differences in the ability of EGF to stimulate hCG production during early pregnancy (Maruo et al., 1992; Genbacev et al., 1994). Our earlier study (Guyda et al., 1990) found that BaP exposure inhibited EGF binding in human placental cell cultures from first trimester, but not term placentas. Thus evidence indicates that developmental windows exist in placental and trophoblast development for altered responses to EGF and chemical toxicity. The differential effect of TCDD and BaP on hCG secretion observed in JEG-3 compared with BeWo cells may reflect developmental differences in the state of differentiation of these two cell lines, with the former being more invasive and proliferative in lower serum conditions (Grümmer et al., 1994; Figure 6-3 and 5). EGF induces differentiation of cytotrophoblasts to form syncytiotrophoblasts and to increase hCG secretion in term placenta (Morrish et al., 1987), with hCG being able to act as an autocrine regulator of further differentiation of cytotrophoblasts (Shi et al., 1993) by enhancing gap junctional communication between trophoblasts (Cronier et al., 1994). Thus, our finding that TCDD and BaP inhibited hCG secretion by BeWo cells suggests that differentiation in this cell line may be adversely affected by both chemicals. In addition, different mechanisms may be involved in the regulation of hCG secretion in these two cell lines, as has been recently reported for JAR human choriocarcinoma cells (Licht et al., 1994).

In summary, BaP treatment resulted in a persistent inhibition of cell proliferation and invasiveness, which may result from disregulation of the balance between positive and negative regulators of trophoblast growth. TCDD exposure did not affect cell proliferation but markedly increased JEG-3 cell invasiveness, suggesting that TCDD may directly shift the balance between expression of proteinases and inhibitors of the proteinases which control cell invasiveness, rather than through alterations in the expression of TGF-α, TGF-β1, c-myc and EGF receptor. In addition, data suggest that placental endocrine function may be affected by TCDD- and BaP-like environmental chemicals during selective periods of placental and trophoblast development.

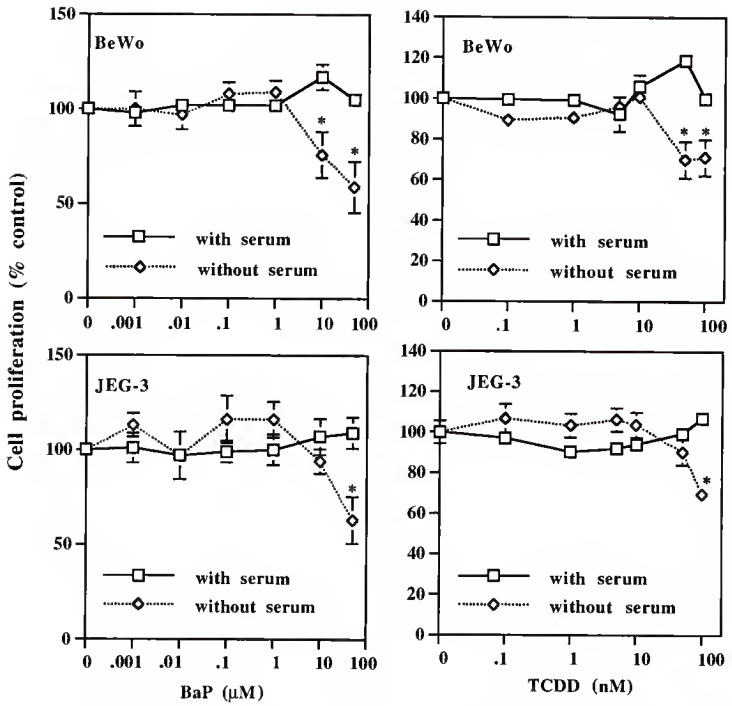


Figure 6-1. Effects of TCDD and BaP on cell proliferation in the presence or absence of FBS. BeWo and JEG-3 cells were cultured at densities of 5 X 10^3 and 2.5 X 10^3 cells/well, respectively, for 20 hr and treated with varying concentrations of TCDD or BaP for another 48 hr in the presence or absence of FBS, respectively. Cell proliferation was determined by the nonisotopic MTT assay. Proliferation of the control cells was set as 100%. Values are the mean ± SE of triplicate cultures from two separate experiments. The points without the standard error bars indicate that the individual SEs are too small to be shown. $*p < 0.05$ as compared to controls by Fisher PLSD and *t* test.

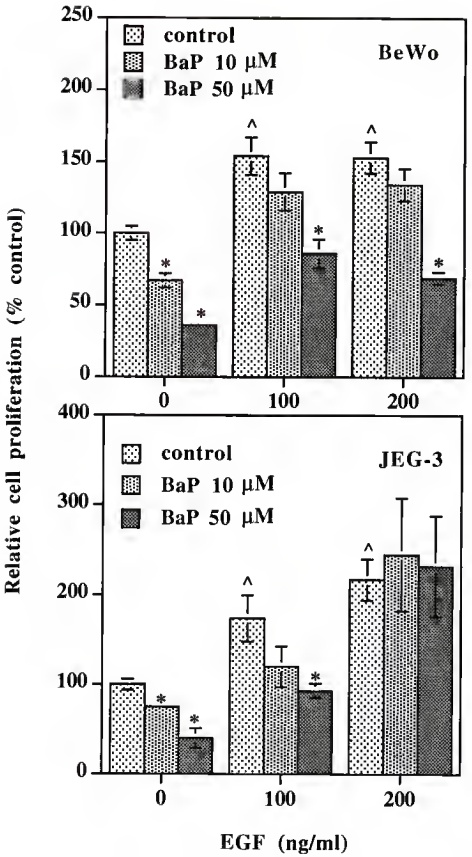


Figure 6-2. Effects of BaP on EGF-stimulated cell proliferation. BeWo and JEG-3 cells were cultured at densities of 5 X 10^3 and 2.5 X 10^3 cells/well, respectively, for 20 hr and treated with BaP and/or EGF for another 48 hr in the absence of FBS. Values are the mean ± SE of triplicate cultures from two separate experiments. Differs from control cells (0 EGF, 0 BaP), $^{\wedge}p < 0.05$; differs from control (0 BaP) in each group, $^{*}p < 0.05$ by Fisher PLSD and *t* test.

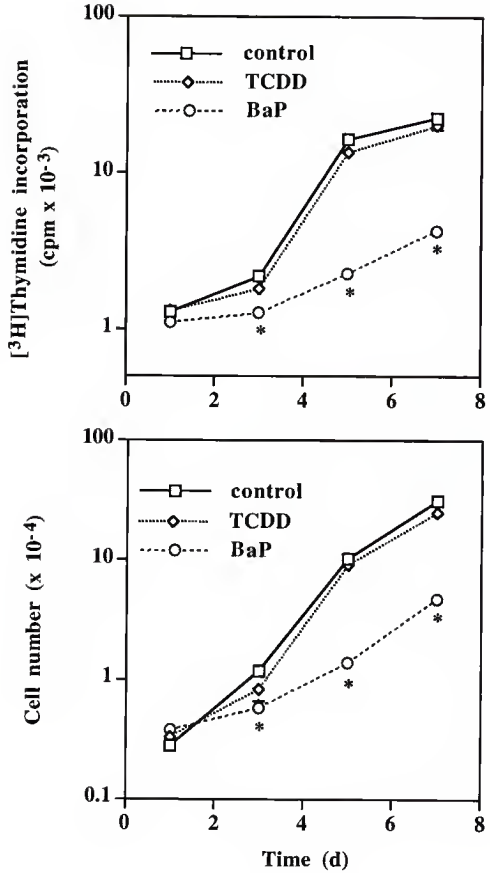


Figure 6-3. Time-dependent effects of TCDD and BaP on JEG-3 cell proliferation. Cells were subcultured at 5.0 X 104 cells/well in 24-well plates for 20 hr, and treated with 10 nM TCDD, 10 μM BaP or 0.1% DMSO in the presence of FBS. [^{3}H]thymidine incorporation and cell number were determined as described in Methods. Cpm values are the mean ± SE of six replicate cultures; cell number values are the mean ± SE of six determinations from duplicate cultures. The points without the standard error bars indicate that the individual SEs are too small to be shown. $*p < 0.05$ as compared with control by t test.

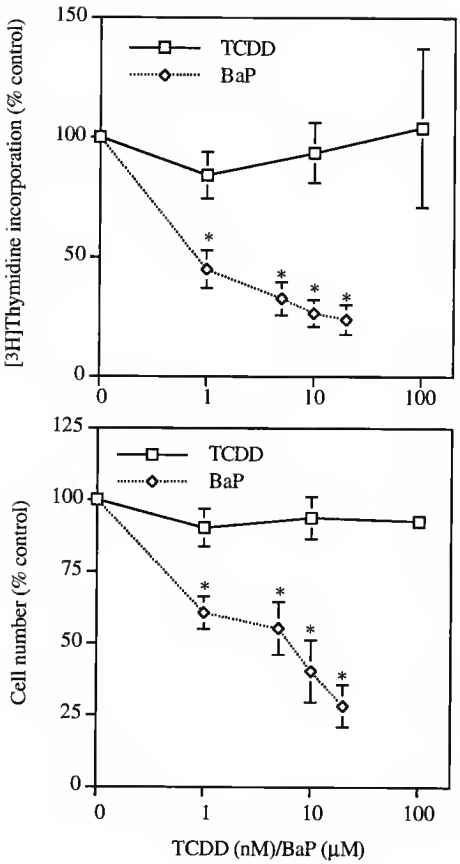


Figure 6-4. Concentration-dependent inhibition of JEG-3 cell proliferation by BaP in the presence of serum. Cells were treated with BaP or TCDD for 5 days, and the incorporation of tritiated thymidine and cell number were determined as desceribed in Methods, with mean cpm and cell number values of control cells being set as 100%. Values are the mean ± SE of three separate experiments. $*p < 0.05$ as compared with control by Fisher PLSD.

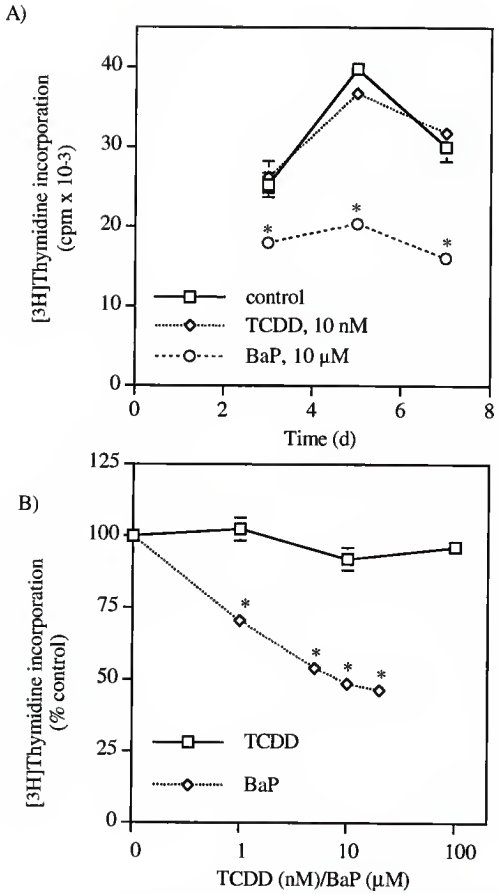


Figure 6-5. Time-course and concentration-dependence of TCDD and BaP effects on BeWo cell proliferation in the presence of serum. A) Time-course; B) Concentration-dependence of cell proliferation following exposure to BaP or TCDD for 5 days. Cells were subcultured at 5.0 X 104 cells/well in 24-well plates, treated with TCDD or BaP as indicated, and the incorporation of tritiated thymidine was determined as described in Methods. Values are the mean ± SE of three separate experiments. The points without the SE bars indicate that the individual SEs are too small to be shown. $*p < 0.05$ as compared with control by Fisher PLSD or *t* test.

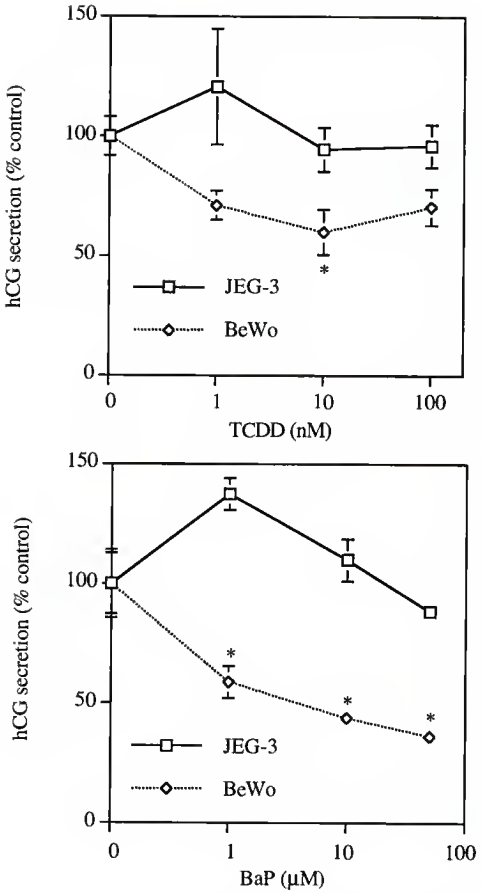


Figure 6-6. Effects of TCDD and BaP on hCG secretion. Cells were exposed to TCDD or BaP for 48 hr, and the conditioned media were collected for assay of hCG levels. Results are expressed as the mean ± SE of three (BaP) or four (TCDD) experiments. $*p < 0.05$ as compared with controls by Fisher PLSD and *t* test.

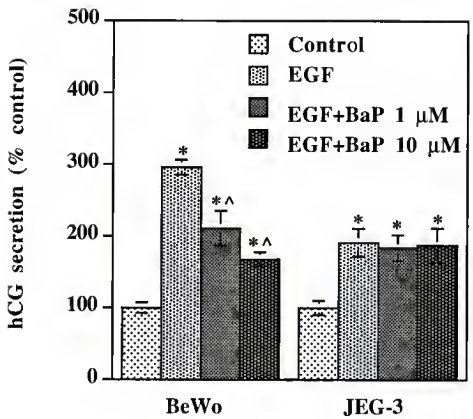
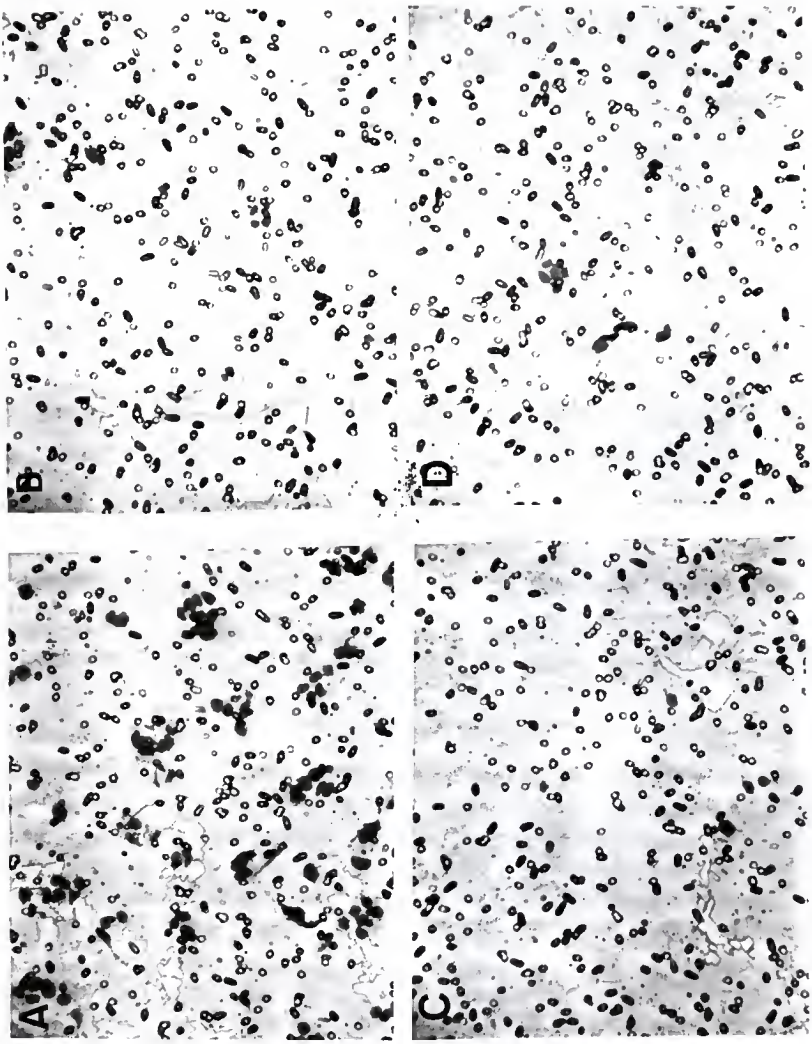


Figure 6-7. Differential effects of BaP-pretreatment on EGF-stimulated hCG secretion. Cells were treated with or without BaP for the first 24 hr in serum-containing medium and then 24 hr in serum-free medium. After being washed three times with Hanks' solution, the cells were exposed to serum free-medium with or without EGF (100 ng/ml, 17 nM) for 24 hr. hCG secretion by control BeWo (123.1 ± 9.2 mIU/ml/24h) and JEG-3 (682.5 ± 67.7mIU/ml/24h) cells was set as 100%. Values are the mean ± SE of six determinations from triplicate cultures. *$p < 0.05$ as compared with untreated (0 EGF, 0 BaP) controls; ^$p < 0.05$ as compared with EGF alone by Fisher PLSD and *t* test.

Figure 6-8. Differential effects of BaP and TCDD on JEG-3 cell invasiveness. A) control; B) BaP, 1μM; C) BaP 10 μM; D) BaP, 20 μM; E) TCDD, 1nM; F) TCDD, 10 nM; and G) TCDD, 100 nM. Cells were pretreated with BaP or TCDD for 48 hr, and then added to the lower compartment of the Boyden chamber at the density of 5,600 cells in 28 μl complete media with the respective chemicals. The upper and lower compartments were separated by a Matrigel-coated Nuclepore filter, and the cells were allowed to invade through the filter at 37°C for 18 hr. The invaded cells were stained with the LeukoStat stain set. (X 200)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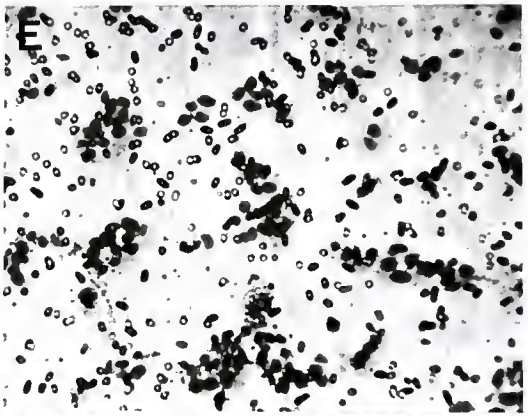

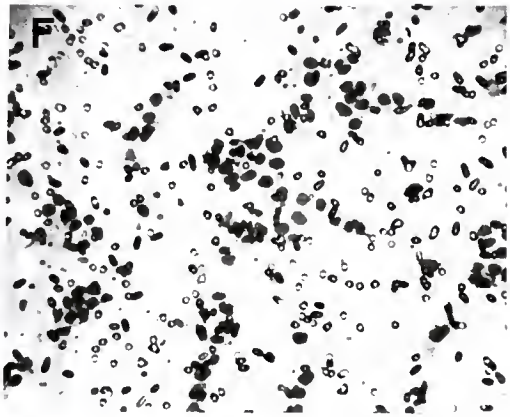

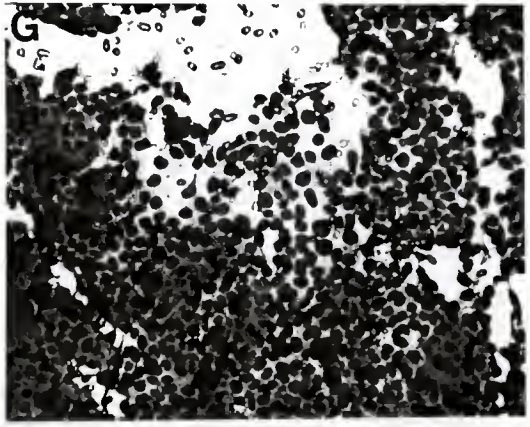

Figure 6-8 continued

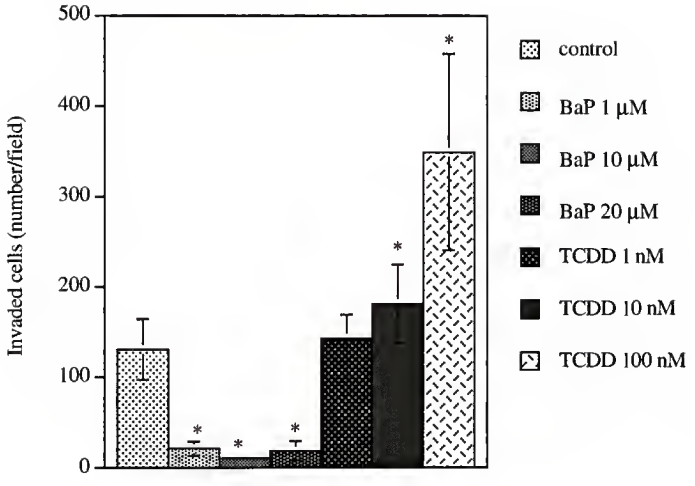

Figure 6-9. Differential effects of BaP and TCDD on JEG-3 cell invasiveness. The invaded cells as shown in Figure 6-8 are counted under the microscope (X 100). Values are the mean ± SE of three experiments. $*p < 0.05$ as compared with control by Fisher PLSD.

CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

BaP is a major polycyclic aromatic hydrocarbon in cigarette smoke, while TCDD is the reference compound for the large group of halogenated aromatic chemicals which are persistent environmental pollutants. Both compounds are known to bind to the Ah receptor and have detrimental effects on fetoplacental development in humans. The objective of this study was to identify the cellular and molecular processes altered by TCDD and BaP in human placental cells, in order to develop an *in vitro* model for the study of placental toxicity of environmental chemicals. The conclusions resulting from these studies, as well as promising areas for future studies, are as follows.

This study has demonstrated that the placental trophoblastic choriocarcinoma cell lines BeWo and JEG-3 express mRNA for Ah receptor and Arnt and respond to TCDD and BaP directly with an induction of CYP1A1 mRNA and protein. Thus, data support the feasibility of using these cell lines as a model to investigate the events which occur during Ah receptor mediated CYP1A1 induction in human placental cells following exposure to the Ah receptor ligands, such as those found in cigarette smoke and PCB mixtures.

Previous studies of pregnant women who smoked cigarettes or consumed PCB-contaminated rice oil found that birth weights were decreased in infants in association with decreased placental EGF receptor binding and tyrosine kinase activity (Lucier et al., 1987; Sunahara et al., 1987; Wang et al., 1988). Hence, the alteration in placental EGF receptors has been proposed as a biomarker of adverse fetoplacental effect for toxic xenobiotics. CYP1A1 is generally accepted as a biomarker of exposure for TCDD and BaP-like compounds (Safe, 1990; Whiltlock Jr., 1993; Hankinson, 1995). This study further investigated the mechanistic link between CYP1A1 induction and EGF receptor

downmodulation. Data have shown that CYP1A1 was induced by both TCDD and BaP in choriocarcinoma cells, but only BaP decreased EGF receptor binding and protein, evidence that does not support a direct causal relationship between CYP1A1 induction and EGF receptor downmodulation. Our data further show that *de novo* mRNA and protein synthesis are required in the BaP-mediated loss of EGF receptor, suggesting that induction of CYP1A1 or another repressor protein may be responsible for the loss of EGF receptors. However, the lack of a specific Ah receptor antagonist which can compete with BaP makes it impossible for this study to clearly show whether the induction of CYP1A1 and loss of EGF receptors are linked, or are independent events following BaP exposure. In the future, the development of better Ah receptor antagonists will serve to more clearly define the role of the Ah receptor in BaP-induced EGF receptor downmodulation. In addition, data indicate that the BaP-mediated loss of EGF receptors does not involve changes in steady state mRNA levels, suggesting that alterations in EGF receptor synthesis, protein processing and half life, or modulation of autocrine networks by BaP may be involved in EGF receptor downmodulation. Future studies aimed at characterizing EGF receptor synthesis, protein processing and half life changes may provide key information on the mechanism of BaP-mediated EGF receptor loss. In this regard, pulse-chase labeling of cells with [^{35}S]methionine would be a useful technique for analyzing the above described time-dependent processes.

Next, this study examined the effects of TCDD and BaP on the expression of the important trophoblast growth control genes TGF-α, TGF-β1, PAI-2 and c-myc. Different patterns of expression were seen in BeWo and JEG-3 cells. These two cell lines may represent distinct developmental stages of the cytotrophoblast, with the JEG-3 cells being more proliferative and invasive (Grümmer et al., 1994). TGF-α mRNA was slightly induced by TCDD in BeWo cells but not in JEG-3 cells. TGF-β1 and c-myc gene expression was markedly altered by BaP in JEG-3 cells. In BeWo cells, however, TGF-β1 mRNA was only slightly increased and c-myc mRNA was not altered following BaP

exposure. These findings suggest that a unique pattern of gene expression may be altered by certain environmental xenobiotics in a given stage of trophoblast differentiation. Moreover, this study has shown that hCG secretion by BeWo cells rather than JEG-3 cells was decreased by TCDD and BaP, with the BaP effect being more profound. The results further support the hypothesis that certain periods during placental development might be especially sensitive to environmental endocrine disruptors. In this regard, fetal development has been shown to be particularly vulnerable to brief periods of endocrine disruption (Kavlock et al., 1996).

The placenta is a dynamic organ which exhibits continuous changes in its growth rate, structure and cellular composition during pregnancy. Patterns of gene expression are known to change as the cytotrophoblast develops into the terminally differentiated syncytiotrophoblast (Ringler and Strauss III, 1990). These developmental changes have major impact on efforts to identify potential biomarkers of placental toxicity. Future experiments, therefore, need to characterize stage-specific responses of the trophoblast to xenobiotics, as well as to assess human risks following exposure to environmental chemicals at a given stage of pregnancy.

This study has further shown that JEG-3 cell proliferation and invasion were inhibited by BaP in association with alterations in EGF receptor, TGF-β1 and c-myc gene expression. In contrast, TCDD was found to increase JEG-3 choriocarcinoma cell invasion without affecting cell proliferation. It is striking that no significant effect was observed on the important trophoblast growth control genes examined in TCDD-treated JEG-3 cells throughout this study. Further experiments are needed to identify alterations in growth factors that stimulate invasion without affecting proliferation, such as IGF-II, MMP-9 and TIMP, which will provide information on the role of growth factors in TCDD-mediated stimulation of invasion. Future studies need to be directed at the identification of expression of ECM proteins, proteases and their inhibitors that directly control trophoblast

invasion, an effort which will aid in understanding the molecular mechanisms by which environmental chemicals disregulate placental invasion and development.

TCDD is resistant to metabolism in all systems studied, while BaP is metabolized by CYP1A1 into a series of reactive metabolites which can lead to a wide variety of toxicity. The observed dissociation between the effects of TCDD and BaP implies that reactive metabolites of BaP or Ah receptor-independent pathways may contribute to BaP-mediated alterations in EGF receptor, TGF-β1 and c-myc expression and in cell proliferation. Future studies focused on the oxidative stress pathway in relationship to BaP metabolism may provide insight into the mechanism by which BaP has more disruptive effects on gene expression than TCDD.

The potential of toxicity caused by BaP metabolites may be biologically important because the inducibility of CYP1A1 has been demonstrated in human placenta as early as the first trimester of pregnancy (Hakkola et al., 1996). Evidence has shown that placental CYP1A1 activity was induced by maternal cigarette smoking and PCB exposure, and the induced activity was able to activate BaP and related polycyclic hydrocarbons into reactive intermediate metabolites, both *in vitro* and *in vivo* (Pasanen and Pelkone, 1994). In this regard, BaP has been detected in tobacco smoke at levels up to 50 ng per cigarette (Hoffman et al., 1978). In JEG-3 cells, induction of CYP1A1 (Figure 3-4) and depression of c-Myc (Figure 5-8) and cell proliferation (Figure 6-4) were observed at 1 μM BaP (250 ng/ml), an amount of BaP which is present in the smoke of only 5 cigarettes. In this regard, previous studies have shown that placentas from women who smoked during pregnancy have increased sister chromatid exchange frequency and smoking-related covalent DNA adducts (Everson et al., 1986; Shulman et al., 1991), and BPDE-, the ultimate carcinogen derived metabolically from BaP, DNA adduct level was found to correlate with higher levels of CYP1A1 activity in the placentas (Manchester et al., 1992).

Altogether, the significance of the findings in this study is that a specific temporal expression of EGF receptor, TGF-α, TGF-β1 and c-myc is important for the control of

trophoblast proliferation, differentiation and invasiveness. The disruption of coordinated expression of these genes may directly interfere with normal placental development, which may consequently lead to fetal growth retardation. Schematic models of trophoblast autocrine/paracrine networks for control of proliferation and invasion and their disruption by TCDD and BaP are illustrated in Figures 7-1 and -2. In addition, these data imply that different mechanisms may be involved in detrimental effects of TCDD and BaP in the human placenta. Data also support the feasibility of using the BeWo and JEG-3 trophoblastic choriocarcinoma cell lines to investigate potential biomarkers and mechanisms of placental toxicity. This approach further serves to identify mechanisms by which individual constituents of cigarette smoke or PCB/PCDF mixtures may directly alter placental function and interfere with fetal growth.

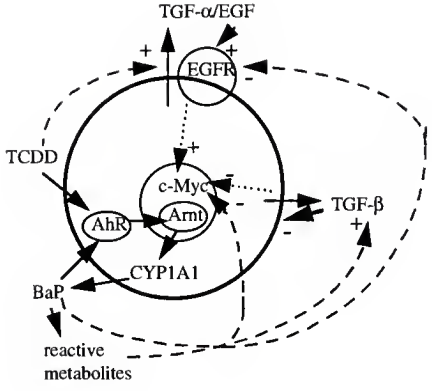

Figure 7-1. Model of trophoblast autocrine/paracrine networks for control of proliferation and their disruption by TCDD and BaP. The growth factors shown are all produced by trohophoblast and decidua cells, and together they form interactive local autocrine/paracrine networks for trophoblast proliferation, differentiation and invasisveness. TGF-α, EGF, EGFR, c-Myc act as positive regulators for trophoblast proliferation. In contrast, TGF-βs are negative regulators of trophoblast proliferation. TCDD increases TGF-α expression, while BaP (and its metabolites) increases TGF-β1 and decreases EGFR and c-Myc expression. Details are in the text of Chapters 1, 3, 4 and 5. + denotes stimulation or increase, and - inhibition or decrease.

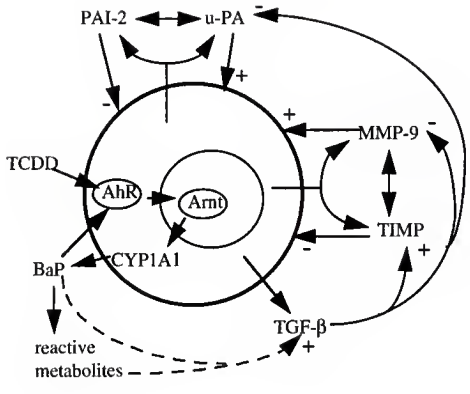

Figure 7-2. Model of trophoblast autocrine/paracrine networks for control of invasion and their disruption by TCDD and BaP. The proteinases and their inhibitors shown are all produced by trohophoblast and decidua cells, and together they form an integrated local autocrine/paracrine network for control of trophoblast invasion. The proteinases u-PA and MMP-9 promote trophoblast invasion. In contrast, TGF-β, PAI-2 and TIMP limit the invasion process. BaP (and its metabolites) increases TGF-β expression, the later then upregulates TIMP and downregulates MMP-9 and u-PA. It remains to be determined how TCDD alters the invasion control network to result in stimulation of invasion. Details are in the text of Chapters 1, 3, 4 and 5. + denotes stimulation or increase, and - inhibition or decrease.

Table 7-1. Summary of the differential effects of TCDD and BaP on choriocarcinoma cells. Prol., proliferation; Inva., invasion. ↑ denotes increase, ↓ decrease, and - no effect.

	CYP1A1	EGFR	TGF-α	TGF-β1	c-myc	Prol.	Inva.	hCG
TCDD	↑	—	↑/–	—	—	—	↑	↓/–
BaP	↑	↓	—	↑	↓	↓	↓	↓/–

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BIOGRAPHICAL SKETCH

Liyan Zhang was born on April 9, 1963, in Zhejiang, China. In 1979, she attended Zhejiang Medical University, in Hangzhou, China, where she received a M.D. degree in 1984. After graduation, she worked as a faculty member in the Departments of Parasitology and Pathophysiology at the Zhejiang Medical University till she came to the United States to pursue further graduate-level study in molecular toxicology in 1992. Since then, she has enrolled in the interdisciplinary toxicology/pharmacology Ph.D. program in the Department of Pharmacology and Therapeutics at the University of Florida, and worked as a graduate research assistant in the field of molecular reproductive and developmental toxicology under the guidance of Dr. Kathleen Shiverick. Upon completion of the Ph.D. program, she will accept postdoctoral training in the area of molecular chemical carcinogenesis in Dr. Mark Steven Miller's laboratory in the Department of Cancer Biology at the Bowman Gray School of Medicine. She hopes to some day use her training and experience in medicine and toxicology to improve people's living environment and quality.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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